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Physiological responses of mycorrhizal tree seedlings to NaCl and other soil factors

by

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Abstract

This dissertation consists of four experimental studies examining effects of different soil chemical and physical properties on mycorrhizal tree seedlings. In Chapter II, ectomycorrhizal black spruce (*Picea mariana*), white spruce (*Picea glauca*) and jack pine (*Pinus banksiana*) seedlings were subjected to KF and NaCl treatments. Fluoride-induced needle injury was alleviated by 60 mM NaCl in black spruce and white spruce, but not jack pine. Chloride tissue concentrations in NaCl-treated plants were not affected by the presence of KF. However, shoot F concentrations in black spruce and white spruce treated with 5 mM KF + 60 mM NaCl were significantly reduced compared with the 5 mM KF treatment. The results point to a possible competitive inhibition of F transport by Cl. In Chapter III, inoculated and non-inoculated jack pine seedlings were subjected to boron and salt treatments. When applied with 60 mM NaCl, 2 mM H_3BO_3 aggravated needle necrosis while reducing Cl concentrations in shoots of non-inoculated plants. Plants inoculated with mycorrhizal fungi had lower shoot Na concentrations compared with non-inoculated seedlings. In Chapter IV, inoculated and non-inoculated American elm (*Ulmus americana*) seedlings were grown in non-compacted and compacted soil and subjected to NaCl treatment. When treated with 60 mM NaCl, ectomycorrhizal seedlings had several-fold lower Na leaf concentrations compared with the non-ectomycorrhizal plants. Soil compaction reduced Na leaf concentrations in non-ectomycorrhizal plants and decreased dry weights, gas exchange and root hydraulic conductance. However, in ectomycorrhizal plants, soil compaction had little effect on the leaf Na concentrations and on other measured growth and physiological parameters. In Chapter V, inoculated and non-inoculated American elm seedlings were subjected to different pH solutions (pH 3, 6 and 9) containing 0 and 60mM NaCl. NaCl treatment reduced leaf chlorophyll concentrations in non-ectomycorrhizal seedlings compared with ectomycorrhizal plants and the greatest decrease occurred at pH 6 treatment. Root Na concentrations were higher in ectomycorrhizal plants at pH 3 treatment compared with non-inoculated seedlings. However, there was no effect of inoculation on root Na concentrations at pH 6 and 9. The results of the above studies point to the importance of soil chemical and physical properties in the responses of mycorrhizal plants to NaCl.

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CHAPTER I: Introduction and Literature Review

1.1 Introduction

In addition to abiotic and biotic environmental stresses that affect plants in their natural ecosystems, human activities have aggravated and added complex stress factors to which the plants are usually not well adapted. Environmental disturbances caused by surface mining and by urban developments have devastating effects on soil physical and chemical properties. Yet, society expects mining areas to be quickly restored into self-sustaining forest ecosystems and urban areas to produce healthy and aesthetically appealing vegetation. Salt is potentially among the most phytotoxic factors that may affect survival and growth of plants in the oil sands reclamation areas and of plants in urban environments. Therefore, this research has focused on the effects of salt, in combination with other soil factors, that are likely to affect plant responses to salt and that are known to be present in the oil sands reclamation and/or urban sites. The soil factors that have been addressed include pH, fluoride, boron and compaction. This study has also attempted to answer the question of a possible beneficial role of mycorrhizal associations in alleviating salt stress in the presence of these soil factors.

Oil sands extraction produces large volumes of tailings, which are deposited in pits. These mining pits must be reclaimed and revegetated with commercially valuable native plant species, such as jack pine (*Pinus banksiana* Lamb.), white spruce [*Picea glauca* (Moench) Voss] and black spruce [*Picea mariana* (Mill.) B.S.P.] (Oil Sands Vegetation Reclamation Committee 1998). In addition to salt, the oil sands reclamation areas may have potentially phytotoxic levels of fluorides and boron and suffer from oxygen deficiency and alkaline pH (Renault et al. 1999).

Salt concerns in urban areas affected by winter snow and ice are largely due to the use of de-icing salts that are spread on roads and sidewalks. The most commonly used salt for road de-icing is NaCl, which may affect roadside vegetation through aerial spray and soil accumulation. In addition to salt, urban soils are frequently characterized by poor water drainage, high pH, various soil and air pollutants and soil compaction due to heavy pedestrian and vehicle traffic (Lait et al. 2001, Kayama et al. 2003, Cunningham et al. 2008). These factors may alter plant responses to salt and affect the development and function of mycorrhizal associations.

Plants resist salt stress by avoiding salt uptake or developing tolerance to elevated salt tissue levels (Levitt 1972, Greenway and Munns 1980). Most of the tree species that are of interest to oil sands revegetation or are planted in urban areas are glycophytes, which are sensitive to salt. However, glycophytes also vary in their sensitivity to salt (Renault 2001, Redfield 2003, Nguyen et al. 2006, Yi et al. 2008) and their ability to regulate the osmotic and ionic stresses (Pludan-Muller 2002). The ability of plants to resist salt stress is compromised by the presence of hypoxia and metabolic inhibitors (Drew and Läuchi 1985, Barrett-Lennard et al. 2003, Apostol and Zwiazek 2003). The presence of ectomycorrhizal associations also alleviates salt stress in plants (Muhsin and Zwiazek 2002a, Bois et al. 2006, Nguyen et al. 2006, Yi et al. 2008). In conifer species such as jack pine, white spruce and black spruce, this improved salt resistance is largely due to the reduction of salt uptake by the shoots of ectomycorrhizal plants (Bois et al. 2006, Nguyen et al. 2006). However, little is known about how soil factors such as boron, fluoride, pH and soil compaction affect mycorrhizal associations and their ability to alleviate salt stress.

Boron is an essential micronutrient for plants (Power and Woods 1997) that is required in tissue concentrations of 5-15 ppm (Kozlowski and Pallardy 1997).

However, boron is phytotoxic in high concentrations (Nable et al. 1997) and may aggravate salt injury in jack pine seedlings (Apostol et al. 2002). The effects of boron and fluoride on mycorrhizal plants have not been extensively studied. Boron is not considered to be an essential element to fungi although it was shown to be absorbed by the ectomycorrhizal fungus *Paxillus involutus* and transported to the host birch tree (Lehto et al. 2004). Little is known about the effects of high concentrations of boron, such as those found in the oil sands tailings (Renault et al. 1999).

Fluoride is one of the most phytotoxic common air and soil pollutants (Fornasiero 2001). It affects various metabolic processes (Zwiazek and Shay 1988, Rakowski and Zwiazek 1992) and water relations (Kamaluddin and Zwiazek 2003, Martínez-Ballesta et al. 2006). Although the effects of fluoride on salt resistance in plants have not been addressed by earlier studies, it is conceivable that, similarly to boron, fluoride may interact with salt to exacerbate its effects on plants.

Since soil compaction reduces soil oxygen content, effects of soil compaction on plants are to some extent similar to that of root hypoxia (Kozlowski 1999). Reduction of root respiration and inhibition of root metabolism may, in turn, affect the ability of roots to sequester salt (Drew and Läuchi 1985, Barrett-Lennard et al. 2003, Apostol and Zwiazek 2003). Soil hypoxia may also affect the ability of ectomycorrhizal associations to alleviate salt stress since soil oxygen deficiency conditions affect mycorrhizal growth and metabolism (Read and Armstrong 1972).

Soil pH is among the most important factors affecting root ion uptake. Plants have limited pH tolerance which can be increased by the presence of mycorrhizal associations (Slankis 1974, Medeiros et al. 1994). Mycorrhizas have been demonstrated to increase growth (Aggangan et al 1996) and root hydraulic conductivity under pH extremes (Siemens 2008). Therefore, it is conceivable that the presence of ectomycorrhizal associations may alter the effects of soil pH on salt responses in plants.

I have investigated the effects of NaCl, in the presence and absence of other stress factors, on mycorrhizal plants. For the studies focusing on the soil factors that are present in the oil sands reclamation areas, I used jack pine, white spruce and black spruce inoculated with different species of mycorrhizal fungi including *Hebeloma sp.*, *Suillus tomentosus* Kauffman, and *Wilcoxina mikolae* var. *mikolae* Yang & Korf. These tree species are among the most common dominant trees of the boreal forest in the northeastern Alberta where the oil sands mining areas are located and these fungi are native to these areas. For the studies addressing soil factors present in urban areas, I selected American elm (*Ulmus americana* L.) as a study tree species and *Laccaria bicolor* (Maire) Orton and *Hebeloma crustuliniforme* (Bull. Ex St. Amans) Quel. as ectomycorrhizal fungi. American elm is considered to be one of the most valuable ornamental tree species in North America (Bey 1990). *Laccaria bicolor* (Kropp and Mueller 2000) and *H. crustuliniforme* (Marmeisse et al. 2000) have low host specificity and are, therefore, likely to form successful mycorrhizal associations with elm (Muhsin and Zwiazek 2002b).

This thesis is divided into four experimental studies with the main objectives to:

1. Study the effects of fluoride on salt resistance of jack pine, black spruce and white spruce seedlings inoculated with *Suillus tomentosus*.
2. Examine the effects of boron on salt resistance in jack pine seedlings inoculated with *Hebeloma* sp., *Suillus tomentosus* and *Wilcoxina mikolae var. mikolae*.

3. Determine how soil compaction affects salt resistance in American elm seedlings inoculated with the ectomycorrhizal fungi *Hebeloma crustuliniforme* and *Laccaria bicolor*.
4. Investigate the effects of soil pH on salt resistance in American elm seedlings inoculated with *Hebeloma crustuliniforme* and *Laccaria bicolor*.

The following hypotheses were examined for each of the four experimental studies:

1. Fluoride would decrease salt resistance of inoculated jack pine, black spruce and white spruce seedlings.
2. The presence of fungal mycelia would increase boron uptake by the host plants both in the presence and absence of salt.
3. Ectomycorrhizal plants would be more resistant to salt stress compared with non-mycorrhizal plants when subjected to soil compaction.
4. Inoculation of plants with ectomycorrhizal fungi would increase tolerance of pH extremes in American elm seedlings and improve their salt stress resistance.

1.2. Literature Review

1.2.1 Biology of the studied tree species

1.2.1.1 Jack pine

Jack pine (*Pinus banksiana* Lamb.) is a widespread tree species of the boreal forest in North America. It is also the northernmost pine species in Canada extending from Nova Scotia to northern British Columbia (Mirov 1967). Jack pine overlaps with lodgepole pine (*Pinus contorta* var. *latifolia* Dougl. ex Loud.) in west-central Alberta and southwestern part of the Northwest Territories, where they hybridize (Moss 1949).

Jack pine is well adapted to short growing seasons and low winter temperatures (Rudolph and Laidly 1990). The trees prefer well-drained loamy soils although they can tolerate drought and low nutrient conditions. Jack pine seedlings typically develop a taproot that may grow up to 3 m into the soil (Rudolph 1958) and which the trees keep into maturity (Rudolph and Laidly 1990). Jack pine is considered to be a pioneer and shade-intolerant species (Cayford et al. 1967). It produces fairly regular crops of seeds every year. The seeds are stored in serotinous cones that typically open after forest fires or extreme temperatures, forming even-aged stands (Cayford et al. 1967). However, jack pine trees can be also found in mixture with other tree species including black spruce (Hosie 1979).

1.2.1.2 Black spruce

Black spruce [*Picea mariana* (Mill.) B.S.P.] trees populate vast areas in the northern parts of North America, including Alberta, typically at altitudes ranging from 150 to 800 m (Lieffers and Macdonald 1990, Viereck and Johnston 1990). Black spruce is a slow-growing, shade tolerant tree that is found in many different forest associations in mixed stands as well as in pure stands that often originate after fire (Viereck and Johnston 1990). Black spruce can survive a wide range of climates and soils including cold, poorly aerated and low nutrient environments (Jeglum and He 1996, Mackenzie and Moran 2004). In waterlogged soils, it produces a shallow root system with abundant lateral roots (Lieffers and Rothwell 1987), which makes the trees prone to uprooting by strong winds (Hosie 1979). Although black spruce often grows in peatlands, its highest productivity is on well-drained mixedwood sites (Viereck and Johnston 1990). Black spruce reproduces by seed and layering (Stanek 1975). High seed crops are produced usually every 2 to 6 years and the seeds are then dispersed from cones throughout the year for several years (Viereck and Johnston 1990).

1.2.1.3 White spruce

White spruce [*Picea glauca* (Moench) Voss] has a wide distribution in the boreal forests of North America, growing from sea level to about 1500 m (Nienstaedt and Zasada 1990). Although white spruce is adapted to a variety of soils, it grows best in a pH range from 4.7 to 7.0 (Nienstaedt and Zasada 1990) and prefers well-drained, moist, silt soils (Hosie 1979). The trees are usually shallow-rooted, but can develop sinker, layered or adventitious multilayered root systems depending on the soil and climatic conditions (Weetman 1962, Caccianiga and Payette 2006).

White spruce is a relatively shade-tolerant conifer that can be found forming even-aged or mixed stands with other conifers or hardwoods (Purdy et al. 2002). White spruce produces the highest seed crops when about 30-years old. The production of high seed crops occurs every 2 to 6 years and the seeds are dispersed throughout fall and winter (Zasada 1985). White spruce can hybridize with

Engelmann spruce (*Picea engelmannii*) and Sitka spruce (*Picea sitchensis*) where their distributions overlap in western Canada (Stiell 1976).

1.2.1.4 American elm

American elm (*Ulmus americana* L.) is a large tree native to central and eastern North America. In Canada, it is naturally distributed from Saskatchewan to Cape Breton Island in the southern part of the boreal forest region, where it grows in either pure or mixed stands (Hosie 1979). The ability of American elm to tolerate urban environments and its high ornamental value make it one of the most valuable urban trees in North America (Bey 1990). However, Dutch elm disease has dramatically reduced the number of these trees in natural forests and urban areas since the disease was first detected in the United States in 1930 (Swinton and Gilligan 2000)

American elm usually grows best in rich, well drained, sandy soils with a surface water table and soil pH from 5.5 to 8.0 (Hosie 1979). The root system has numerous short lateral roots, which frequently contains vesicular-arbuscular (VA) Paris-type mycorrhizal associations (Brundrett et al. 1990). The trees reproduce by seeds that are dispersed by wind in spring. When mature, American elm trees are very prolific producers of seeds (Bey 1990).

1.2.2 Biology of the studied mycorrhizal fungi

Mycorrhizal fungi form mutualistic associations with plants and may assist survival of their hosts in areas affected by different abiotic stresses. In addition to supplying trees with essential nutrients (Marschner and Dell 1994, Smith and Read 1997), mycorrhizas enhance water uptake (Landhäusser et al. 2002, Muhsin and Zwiazek 2002a,b) probably largely by enhancing the expression of water-conducting root aquaporins (Marjanović et al. 2005). Mycorrhizal associations also help alleviate metal toxicity (Jones and Hutchinson 1988, Jentschke and Godbold 2000), salt stress (Bois et al. 2006, Nguyen et al. 2006, Yi et al. 2008), effects of soil pH extremes (Kernaghan et al. 2002, Siemens 2008), and protect plants against pathogens (Morin et al. 1999, Robin 2001). In exchange, fungi obtain carbohydrates from their plant hosts (Simard and Durall 2004) and may form a network of mycelia between trees of the same and different species (Simard and Durall 2004, Philip and Simard 2008).

Although mycorrhizas have been commonly reported to enhance plant growth (Nguyen et al 2006, Karst et al. 2008), they have been also reported to decrease (Corrêa et al. 2006, Nguyen et al 2006) or have no effect (Landhäusser et al. 2002, Corrêa et al. 2006, Yi et al. 2008) on plant growth. The effect of mycorrhizas may depend on the compatibility of plant-fungi species and the age and nutritional status of the plant (Jones and Smith 2004, Corrêa et al. 2006).

Conifer species are typically ectomycorrhizal (ECM) but some species in the *Pinus*, *Picea*, *Larix* and *Populus* genera have been found to also form ectendomycorrhizal (Trevor et al. 2001, Peterson et al. 2004, Siemens 2008) and arbuscular mycorrhizas (Cázares and Smith 1996, Wagg et al. 2008). Most often, ectomycorrhizas involve fungi of the *Basidiomycota*, although ascomycetes are also very important ECM fungi (Egger 2006, Tedersoo et al. 2003). The ECM associations are characterized by the formation of a Hartig net of hyphae distributed between the root cells, a mantle of fungal hyphae covering lateral roots and hyphae growing from the mantle into the surrounding soil (Peterson et al. 2004). The exchange of water and nutrients between the plants and the fungus occurs in the apoplastic interface of secondary and tertiary roots (Peterson and Massicotte 2004)

Ectendomycorrhizas resemble ectomycorrhizas: they also form a mantle and Hartig net, but the fungal hyphae can also penetrate cortical cells (Trevor et al. 2001). Fungal species within the *Wilcoxina* genus have been reported to form ectendomycorrhizal associations with some tree species, while forming ECM associations with other species (Trevor et al. 2001, Peterson et al. 2004, Siemens 2008).

1.2.2.1 Suillus

Suillus species (*Basidiomycota*) form ECM associations and demonstrate a high degree of host specificity with *Pinaceae* of the northern temperate and boreal forests (Bois et al. 2006, Danielson 1984). The most common tree genera associated with *Suillus* are *Pinus, Larix* and *Pseudotsuga* (Dahlberg and Finlay 1999). These fungi have a high sporocarp production that can alter the abundance of fungal species in ECM communities at different forest successional stages (Dahlberg and Finlay 1999), being present from young to mature stands (Danielson and Visser 1989, Danielson 1991). The mycorrhizas are dichotomously branched to tuberculate forms and are characterized by the presence of profuse mycelia and conspicuous rhizomorphs (Dahlberg and Finlay 1999).

1.2.2.2 Laccaria

Laccaria species (*Basidiomycota*) have been found in almost every continent and are quite common in North America. They are often regarded as ECM fungi with low host specificity; however, some species of *Laccaria* display limited geographic ranges and have some degree of host specificity (Mueller 1992). *Laccaria* species are considered to be pioneer fungi and are frequently found in disturbed sites, after fires and in young forest stands (Danielson 1984, Kropp and Mueller 2000). *Laccaria* is widely used in nurseries and for field and laboratory studies due to its fast growth and relatively simple growth requirements. The macromorphology of the mycorrhiza varies with the host tree species (Kropp and Mueller 2000). In general, *Laccaria* mycorrhizas have a consistent Hartig net that extends through the cortex and a well-defined mantle with clamped, undifferentiated, hyphae (Mueller 1992).

1.2.2.3 Hebeloma

Hebeloma species (*Basidiomycota*) are ECM fungi that are well represented in North America, Europe and Asia. *Hebeloma* species have a broad host distribution and can be associated with various species of angiosperm and gymnosperm trees (Kernaghan and Currah 1998). Several species of *Hebeloma* appear in the early stages of ECM fungal succession and, hence, may represent pioneer species. They are dominant species associated with young trees and tree seedlings and occupy the periphery of the root system in older trees (Bruchet 1970).

Hebeloma mycorrhizas usually posses a well-developed mantle and a Hartig net surrounding the cortical cells. Polyphosphate granules and glycogen are common in the hyphae of the Hartig net (Marmeisse et al. 1999). *Hebeloma crustuliniforme* has been used for studies with a wide range of host tree species and is of potential interest as a commercial inoculum for forestry purposes.

1.2.2.4 Wilcoxina

Wilcoxina species (*Ascomycota*). can form ectomycorrhizal or ectendomycorrhizal associations depending on the host plant species (Trevor et al. 2001). They are widely distributed and associate with a wide range of plant hosts (Molina et al. 1992). It has been reported that *Wilcoxina mikolae* and *W. rehmi* form ectendomycorrhizal associations with *Pinus, Picea, Larix* (Trevor et al. 2001, Scales and Peterson 1991a, Wagg et al. 2008) and *Populus* (Siemens 2008). Ectomycorrhizal associations of *Wilcoxina* have been reported for *Picea mariana* and *Betula alleghaniensis* (Scales and Peterson 1991b), *Pinus sylvestris* (Aučina et al. 2007) and *Picea abies* (Rudawska et al 2006). Pine-*Wilcoxina* ectendomycorrhizas are characterized by the formation of dichotomous short roots with a thin mantle and sparse extraradical hyphae (Peterson 2004).

Wilcoxina spp. have been commonly reported in tree nurseries (Hagerman et al. 1999, Mah et al. 2001, Izzo et al. 2006, Rudawska et al. 2006). In nature, *Wilcoxina* is commonly found in young forest stands (Jones et al. 2003, Fujimura et al 2005, Hart et al. 2005). They are believed to play an important role for the establishment of conifer seedlings and for the fungal succession in disturbed areas (Danielson 1991, Trevor et al. 2001).

1.2.3 Effects of studied soil factors on plants

1.2.3.1 Salinity

Although different types of salt can cause injury to vegetation, NaCl is the most widespread phytotoxic salt affecting plants in various types of environments (Munns and Termaat 1986, Renault 1999, Franklin 2002). Salt accumulates naturally at the surface of some soils in arid and semiarid regions where there is insufficient rainfall to flush it from the upper soil layers (Brandy and Weil 2000). Human activities such agricultural irrigation, application of de-icing salts in urban areas and mining activities can locally increase soil salt levels. Salt becomes progressively concentrated in the root zone of plants and induces water stress, nutrient imbalance and ion toxicity (Munns 1993, Czerniawska-Kusza et al. 2004, Franklin and Zwiazek 2004).

Organisms survive saline environments by tolerating or avoiding salt stress (Levit 1972). Halophytes are plants that can tolerate high concentrations of salt by different mechanisms such as accumulating salt in the tissues and using it to balance the osmotic potential of the soil solution (Greenway and Munns 1980), excreting excess of salt through specialized glands from the leaves (Jacoby 1994) or by compartmentalizing Na and Cl within the cells (Hasegawa et al. 2000). There are very few forested areas with elevated salt levels (Lieffers 1984, Schwarz and Wein 1997) and most of the forest plants are regarded as salt-intolerant glycophytes. The level of salt resistance varies amongst tree amd shrub species (Renault 2001, Redfield et al. 2003, Purdy et al. 2005, Bois et al. 2006, Nguyen et al. 2006, Yi et al. 2008) and grasses (Renault et al. 2004). Salt resistance of glycophytes is believed to be related to their ability to avoid high salt concentrations in sensitive areas of the plant (Hagermeyer 1997).

The presence of high concentrations of NaCl in plants produces osmotic and ionic stresses. A decrease in the osmotic potential in the presence of salts results in an osmotic imbalance within the plant. These osmotic and ionic stresses may induce needle injury (Franklin et al. 2002), reduction in photosynthesis and stomatal closure (Redfield and Zwiazek 2002, Apostol et al. 2002, Nguyen et al. 2006) as well as a decrease in water uptake (Rodriguez et al. 1997). In addition, NaCl is a strong inhibitor of water channel proteins (aquaporins) (Carvajal et al. 2000, Martínez-Ballesta et al. 2000, López-Berenguer et al. 2006) and this will interfere with water and nutrient uptake (Shannon 1997), hamper cell membrane permeability (Franklin and Zwiazek 2004, Salama et al. 2007), and alter enzyme activities in cells (Greenway and Munns 1980, Munns 1993, Franklin et al. 2002).

Sodium accumulates first in the roots of conifer trees (Franklin and Zwiazek 2004) and then appears to move to the shoots after Na concentrations exceed a certain threshold level (Franklin and Zwiazek 2004, Bois et al. 2006, Nguyen et al. 2006). With some deciduous tree species (Yi et al. 2008), and with plants dependent on roots for reproduction (Redfield et al. 2003), the trend appears to be the opposite, with higher accumulation of Na in leaves than in roots. Sodium influx into the cells occurs through permeable transporters (Roberts and Tester 1997, Tyerman et al. 1997) and Na is sequestrated into the vacuoles of cells by Na^+/H^+ antiporters (Blumwald and Poole 1985) allowing the cytoplasm to maintain turgor and the enzymatic activities (Glenn et al. 1999).

Chloride accumulates in greater amounts than Na in the shoots (Jacoby 1994, Franklin and Zwiazek 2004, Nguyen et al. 2006, Yi et al. 2008) and it is likely the main factor responsible for the initial needle injury in conifer seedlings (Franklin and Zwiazek 2004). Chloride is the main factor contributing to the loss of membrane integrity (Bernstein 1975, Redfield and Zwiazek 2002, Franklin and Zwiazek 2004) and reduced capacity of roots for Na sequestration in salt-treated plants (Bernstein 1975, Redfield and Zwiazek 2002, Franklin and Zwiazek 2004). Chloride is transported across the plasma membrane via an active process (Maas and Ogata 1972, Tyerman and Skerrett 1999). In jack pine seedlings, the uptake and transport of Cl is independent of external concentration (Franklin and Zwiazek 2004), suggesting that rates are limited by the presence of chloride channels in the plasma membranes.

1.2.3.2 Boron

Boron is an essential plant micronutrient that usually appears in the soil in small quantities (Goldberg 1997).). Boron is absorbed by roots as boric acid (H_3BO_3) and is involved in cell wall structure and function (Hu and Brown 1994), enzyme activation, and carbohydrate transport (Loomis and Durs 1992, Power and Woods 1997).The amount of boron required for maintaining tissue functions in woody plants is about 20 mg kg^{-1} dry weight (DW) (Renault et al. 1999, Fitter and Hay 2002). High B concentrations affect plants by inhibiting root growth (Apostol et al. 2002) and photosynthesis (Lovatt and Bates 1984, Nable et al. 1997). Boron usually accumulates in the leaves producing chlorotic and necrotic lesions of leaf tips and margins (Brown and Shelp 1997, Apostol and Zwiazek 2004).

High B concentrations are often associated with saline soils (Gupta et al. 1985, Renault et al. 1998) and are affected by human activities including irrigation and surface mining (Nable et al. 1997). The ability of plants to tolerate elevated soil B levels largely depends on the restriction of uptake, sequestration in the cell walls and cytoplasm of root cells, and retranslocation of B to less sensitive parts of the plant (Nable 1997). Until recently, B was believed to have restricted mobility in plants (Brown and Shelp 1997), however, the degree of B mobility varies between species (Brown and Shelp 1997). Boron has been shown to be mobilized through boron-polyol complexes in coniferous trees (Lehto et al. 2004), coffee (Leite et al. 2007) as well as celery and peach (Hu et al. 1997).

The exact mechanisms of B uptake by plant roots are not fully understood, however, it is believed that it is affected by the transpiration intensity (Raven 1980) and may be energy-demanding (Gassert et al. 2002). The mechanism of transmembrane transport is largely unknown, but may involve aquaporins (Dordas and Brown 2001, Bastías et al. 2004). Since mycorrhizal fungi have been shown to increase the expression of aquaporins (Marjanović et al. 2005), it is plausible that they may also affect B uptake and transport in plant roots. Boron is not considered to be an essential element to fungi although it has been shown to be absorbed by the ectomycorrhizal fungus *Paxillus involutus* and transported to the host birch tree (Lehto et al. 2004).

1.2.3.3 Fluoride

Fluoride is a common air and soil pollutant that is emitted during various industrial processes. Fluorides easily combine with hydrogen and soluble salts forming extremely dangerous and poisonous compounds for plants, fungi and animals (Formasiero 2001, Weinstein and Davison 2004). The uptake of soil fluoride depends on the concentration of soluble fluoride in soil, the capability of the soil to replenish fluoride in the soil solution, soil pH, and the content of clay and the organic matter (Thomas and Alter 1966). Fluoride is transported in plants as F^- or HF with the transpiration stream and accumulates in the leaf tips and margins causing leaf necrosis and chlorosis (Weinstein and Davison 2004). The concentration of fluoride in leaves that can cause visible injuries is less that 50 ppm for very sensitive plants, ,50 to 200 ppm for sensitive ones and more than 200 ppm for resistant ones (Brandt 1971).

Physiological responses to F are the reduction of photosynthetic and transpiration rates (Zwiazek and Shay 1988, Rakowski and Zwiazek 1992), and respiration (Zwiazek and Shay 1988, Kamaluddin and Zwiazek 2003), and an inhibition of aquaporin-mediated water transport in roots (Kamaluddin and Zwiazek 2003, Martínez-Ballesta et al. 2006). The pathway of fluoride tissue transport in plants is thought to be largely apoplastic (Drury et al. 1980, Takmaz-Nisancioglu and Davison 1988, Weinstein and Davison 2004). The mechamism of transmembrane transport of F is little studied, but may involve anion channels (Chapman and Kuchel 1990) and aquaporins. It has been also suggested that F is transported across cell membranes partly in an uncharged form of HF (Kronberger 1988).

Fluoride is a strong inhibitor of plasma membrane H^+-ATPase (Giannini et al. 1987, Rakowski et al. 1995) and alters phosphorylation of membrane proteins (Chang and Kaufman 2000, Struglics et al. 2000). Furthermore, some Cl channels in plasma membranes and tonoplast in plants may have a higher specificity for F than for Cl (Skerrett and Tyerman 1994, White and Broadley 2001). Therefore, the presence of F may alter the transmembrane transport of Cl (Giannini et al. 1987).

1.2.3.4 pH

pH is a measure of the H^+ concentration in the soil solution. The main factors affecting soil pH are the chemical composition of parent materials (Brandy and Weil 2000), associated plant and microbial species (Epstein and Bloom 2005), and the presence of industrial pollutants (Goulding et al. 1998). In Alberta, pH values of 6-8 are typical for agricultural soils, pH 3.6-7.5 for forest peat soils, pH 4.8-6 for mineral soils, pH 5.7-9.3 for oil sands-reclamation materials and pH 5.6-5.9 for the rain water

(Howat 2000). The soil pH may reach values as high as 9 in some urban sites in Edmonton (Calvo Polanco and Zwiazek, unpublished data).

Acidic soils are usually associated with humid environments and alkalinity with the arid ones (Brandy and Weil 2000). Increasing adsorbed Al^{3+} and H^+ together with lower availability of Ca^{2+}, Mg^{2+} and K^+ are largely responsible for plant growth problems in acidic soils (Brandy and Weil 2000, Larcher 2003). Alkaline soils are saturated with Ca^{2+}, $Mg^{2+}$and Na^+ and are characterized by low availability of Fe, Mn, P and Zn (Yang et al. 1994, Valentine et al. 2005). Alkaline pH, unlike acidic pH, is a determinant in plant species distribution (Anderson and Davis 1997, Locky and Bayley 2006) and plant community succession, in association with climate (Prach et al. 2007).

The pH tolerance of plants varies between plant species, but most plants are able to survive soil pH between 3.5 and 8.5 (Illes 2001, Larcher 2003). Plant roots can acidify their rhizosphere to increase solubilization and availability of nutrients (Nelson 1992, Bernal and McGrath 1994). Plants may modify pH of the rhizosphere and increase alkalinity tolerance by extruding H^+ through the plasma membrane H^+-ATPase (Jahn and Palmgren 2002). The optimum pH for the plasma membrane H^+-ATPase activity is usually about 6.5 and may be affected by the lipid composition of the membrane (Palmgren 1988). Since membrane structure and composition may be altered by the external pH, H^+-ATPases with different pH optima could play a role in conferring plant tolerance to acidity or alkalinity (Axelsen et al. 1999, Luo et al. 1999). Furthermore, extreme acid or basic pH conditions can reduce root water flux (Tang et al. 1993, Ktitorova et al. 1998, Kamaluddin and Zwiazek 2004) that may be related to changes in aquaporin activity (Voicu and Zwiazek 2004, Aroca et al. 2006).

Mycorrhizal associations can alter the pH tolerance of plants (Slankis 1974, Medeiros et al. 1994) and increase growth (Aggangan et al 1996) and root hydraulic conductivity under pH extremes (Siemens 2008). Additionally, mycorrhizal roots may act as a rhizospheric pH buffer via H^+/OH^- extrusion (Rygiewicz et al. 1984a, Rigou et al. 1995, Bago and Azcon-Aguilar 1997). Thus, mycorrhizae may contribute to the pH-buffering capacity of roots, producing a greater pH tolerance of their plant hosts, and should be considered as an important factor for soil pH resistance of plants.

1.2.3.5 Soil compaction

Soil compaction is quite common in urban and forested areas, particularly in sites that are affected by vehicle traffic and heavy equipment. Compaction affects the physical properties of soil by decreasing pore size and distribution (Greacen and Sands 1980, Gregory et al. 2006). Soil compaction affects water movement in the soil and oxygen availability for root growth and development (Haeussler and Kabzems 2005; Mariani et al. 2006).

Among the main effects of soil compaction, root hypoxia (oxygen deficiency) is of major concern for tree survival. Hypoxia affects all energy-demanding processes in roots and reduces root and shoot growth (Huang et al. 1994, Pardo et al. 2000, Apostol and Zwiazek 2003), inhibits photosynthetic and transpiration rates (Drew 1983, Islam and Macdonald 2004, Kogawara et al. 2006) and reduces root hydraulic conductivity (Everard and Drew 1989, Kamaluddin and Zwiazek 2001). The effects of hypoxia may also include reducing the ability of plants to sequester ions (Drew and Läuchi 1985, Apostol and Zwiazek 2003). Hypoxia affects the energy available for the activity of plasma membrane H^+-ATPases (Shen et al. 2006) and ATP content in root cells (Vartapetian and Jackson 1997) that are crucial for plant survival (Shen et al. 2006). The reduction of the activity of H^+-ATPases under hypoxic conditions may affect the electrochemical gradients that drive the ion transport across cell membranes as well as inhibit the function of root aquaporins that may result in a reduction of root water uptake (Zhang and Tyerman 1991, Kamaluddin and Zwiazek 2002).

Soil oxygen deficiency conditions can also affect mycorrhizal growth and metabolism (Read and Armstrong 1972). Different studies have shown growth improvement in plants under soil compaction in the presence of mycorrhizal fungi (Yano et al. 1998, Miransari et al. 2007). Mycorrhizas of trees are highly oxygen demanding (Slankis 1974). However, mycorrhizal fungal hyphae can occupy smaller soil pores than the finest plant roots (Allen 2007) and may be able to find resources in the areas not accessible by roots. The presence of mycorrhizal fungi in plant roots may also increase tolerance to hypoxia by improving plant nutrition and inhibiting ethanol accumulation in roots (Rutto et al. 2002).

1.2.4 Oil sand reclamation areas

Large-scale surface mining operations of bitumen present in oil sands have severely impacted vast forested areas in the northeastern Alberta since major commercial production started in 1967. The area is part of the central mixedwood section of the boreal forest region with dominant tree species that include trembling aspen (*Populus tremuloides*), balsam poplar (*Populus balsamifera*), white spruce (*Picea glauca*), black spruce (*Picea mariana*), and jack pine (*Pinus banksiana*) (Stringer 1976). The plan for long-term reclamation of the mining areas includes a mosaic of forests, grasslands, wetlands and lakes.

Oil sands ore contains small amounts of NaCl that become more concentrated with the extraction and production of process waters. The extraction of bitumen from the ore is based on a hot water procedure resulting in the production of large volumes of solid (tailings sand) and aqueous (fine) tailings. One of the present options for tailings management involves their treatment with gypsum (Matthews et al. 2000). The gypsum can settle the solid particles of the fine tailings and make the deposited tailings suitable for reclamation (MacKinnon 1998). The resulting tailings are known as "consolidated" (Syncrude Canada Ltd.) tailings that can upport the transportation of vehicles in one to two years. However, the treatment with gypsum replaces Na from clay particles and increases salinity of the tailings (FTFC 1995). In addition to Na, the consolidated tailings contain high levels of Cl, SO_4, B and F and have pH of 7.8 to 8.5 (FTFC 1995, Renault et al. 1998, Franklin and Zwiazek 2002).

1.2.5 Urban areas

Urban areas are fragile and challenging ecosystems to plants. Urban soil may be compacted, have poor water drainage and high pH, and contain high levels of deicing salts and various soil and air pollutants (Cain et al 2000, Malmivaara-Lämsä et al. 2008). Since urban trees have high esthetic, recreational and economic value, their protection is a major issue to the society.

Extremely hostile soil conditions to plant growth are created by the use of deicing salts on city roads (Lait et al. 2001, Kayama et al. 2003, Czerniawska-Kusza et al. 2004, Cunningham et al. 2008) during the winter. The common agent used for most roads is NaCl with some supplement of $CaCl_2$ (Werner and diPretoro 2006). In regions with very cold winters, the amount of salt used in roads has been increasing in recent years with the increasing of the average temperatures during the winters to a range where salt is an effective de-icing agent (Jackson and Jobbagy 2005). This can negatively affect roadside vegetation, streams and aquifers (Hutchinson 1970, Roth and Wall 1976). It has been estimated that over $1 billion is spent annually in Canada on snow and ice control of roads (TAC/ATC 2003). The amount of salt used to increase the road safety during winter conditions is a function of local policies, practices, roadway system, funding constraints, and weather conditions (TAC/ATC 2003).

Roadside vegetation may be injured by exposure of shoots to salt spray or from salt accumulating in the soil and taken up by roots (Kayama et al. 2003, Paludan-Muller et al. 2002). There is little information concerning the effects of airborne salt on trees; however, in some locations in Ontario, elevated salt levels were measured in areas as distant as 3 km from the source of road salt (Cain et al. 2000). Salt is not the only stress that plants have to deal in urban areas. Deicing salt may affect urban plants growing in soils that are compacted and have a pH that is not favorable to plant growth. In some urban sites, soil compaction can reach the values close to 1.4 kg cm^{-3} and pH of 9 is not uncommon (Calvo Polanco and Zwiazek, unpublished data). In addition to high mortality, tree injuries such as chlorosis and necrosis are commonly observed in urban trees and the reductions of photosynthesis and growth have been frequently reported (Viskari and Karenlampi 2000, Kayama et al. 2003, Calvo Polanco and Zwiazek, unpublished data). It has been estimated that the City of Edmonton lost about 23,000 trees due to drought and salt between 2002 and 2006 (Milton Davies, City of Edmonton, personal communication).

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CHAPTER II: Effects of NaCl on responses of ectomycorrhizal black spruce (*Picea mariana*), white spruce (*Picea glauca*) and jack pine (*Pinus banksiana*) to KF

2.1 Introduction

Fluorine is an abundant element that is present in soil and water in the form of fluorides. Most fluorides are insoluble in water and, therefore, they are not readily absorbed by plants (Weinstein and Davison 2004). However, when present in an ionic form of F^- or as HF, fluoride is highly phytotoxic (Fornasiero 2001). In addition to natural sources, various industrial processes result in F emissions which can induce injuries and impede the growth of plants (Weinstein and Davison 2004). Elevated levels of soluble F and NaCl have been found in tailings that are produced as a result of oil sands extraction in northeastern Alberta, Canada (Renault et al. 1998, Franklin et al. 2002). These tailings are deposited in oil sands mining areas and are revegetated with local plant species including *Picea mariana* (Mill.) B.S.P., *Picea glauca* (Moench) Voss and *Pinus banksiana* Lamb.

Fluoride can be absorbed by leaves from the air or by roots from the soil. It is transported within plants via the transpiration stream and accumulates in leaf tips and margins causing leaf necrosis and chlorosis (Weinstein and Davison 2004). Although the exact pathway of fluoride tissue transport in plants is not clear, it is thought to be largely apoplastic (Drury et al. 1980, Takmaz-Nisancioglu and Davison 1988, Weinstein and Davison 2004). The sites of transmembrane transport of fluoride in plants are largely unknown, but they may involve the anion channels that are involved in Cl transport (Chapman and Kuchel 1990).

Physiological effects of F include reductions in photosynthetic and transpiration rates (Zwiazek and Shay 1988, Rakowski and Zwiazek 1992), respiration (Zwiazek and Shay 1988, Kamaluddin and Zwiazek 2003) and altered water relations (Kamaluddin and Zwiazek 2003). Fluoride is a strong inhibitor of many enzyme systems including plasma membrane H^+-ATPase (Giannini et al. 1987, Rakowski et al. 1995) and alters phosphorylation of membrane proteins (Chang and Kaufman 2000, Struglics et al. 2000). This inhibition may alter the aquaporin-mediated transport of water in plants (Kamaluddin and Zwiazek 2003). Fluoride was also demonstrated to competitively interact with Cl stimulation of proton transport and to inhibit the uptake of radioactive chloride into sealed vesicles (Giannini et al. 1987).

Similarly to F, an accumulation of Na and Cl above threshold tissue levels triggers plant injury in plants exposed to salt. Numerous studies have demonstrated that plant uptake and tissue concentrations of Na and Cl can be reduced and their salt tolerance increased by the presence of ectomycorrhizal (ECM) associations (Muhsin and Zwiazek 2002, Bois et al. 2006, Nguyen et al. 2006, Langenfeld-Heyser et al. 2007). However, since F inhibited growth of some pathogenic fungi (Threshow 1965), it is also possible that it may affect ECM interactions with plants and thereby interfere with the ability of ECM plants to resist salt stress. On the other hand, since F may interfere with the membrane transport of Cl (Giannini et al. 1987), it could also potentially affect Cl uptake and salt injury.

I examined the hypothesis that KF would alter NaCl resistance of ECM jack pine, black spruce and white spruce seedlings. The seedlings were inoculated with *Suillus tomentosus* which was shown in an earlier study to reduce NaCl injury to *P. banksiana*. *Suillus spp.* are ECM fungi that exhibit a high degree of host specificity to conifers and their distribution overlaps with the natural distribution of *Pinaceae* in the northern hemisphere (Dahlberg and Finlay 1999).

2.2 Materials and Methods

2.2.1 Plant material and growth conditions

Seeds of black spruce (*Picea mariana*), white spruce (*Picea glauca*) and jack pine (*Pinus banksiana*) were collected from trees growing in the boreal forest near Fort McMurray, AB, Canada. The seeds were sterilized with 5% H_2O_2 for 1 min and germinated in Petri dishes on moist sterilized sand. One week after germination, the seedlings were transplanted to 170 ml Spencer-Lemaire root trainers (Spencer-Lemaire Industries Ltd. Edmonton, AB, Canada) containing a sterilized mixture of peat moss and sand (2:1, by volume). Before filling the pots, the substrate was autoclaved at 121°C for 20 min with a 24-h interval. The pots with seedlings were placed in a controlled-environment growth room under the following environmental conditions: 22/18°C day/night temperature, 16-h photoperiod, 65±10% relative humidity, and 300-350 μmol m^{-2} s^{-1} photosynthetic photon flux density at the seedling level. The seedlings were provided with Hoagland's mineral nutrient solution (Epstein and Bloom 2005) once per week. For the last two weeks before fungal inoculation, the concentration of Hoagland's solution was lowered to 25% (v/v). The soil was flushed weekly with de-ionized water to prevent ion accumulation.

2.2.2 Fungal inoculation

When four-months old, the seedlings were inoculated with *Suillus tomentosus* (Kauffman) Singer, Snell & Dick). Pure culture of *S. tomentosus* (UAMH 5506), collected in northwestern Alberta, Canada, was obtained from the University of Alberta Microfungus Collection and Herbarium (UAMH). Prior to inoculation, the culture was grown in a modified Melin-Norkans (MMN) liquid medium (Mason, 1980) for four weeks as previously described (Calvo Polanco et al. 2008). Seedlings were inoculated by applying 20 ml of the fungal culture with a sterile pipette to the surface of the root plugs after opening the root trainers.

2.2.3 Root fungal colonization

Once the physiological measurements started, six seedlings per plant species per treatment combination (n=6) were randomly taken to determine fungal colonization. Six distal root segments, 1-2-cm long, were removed from each root system and placed in a formalin-acetic acid-ethanol (Ruzin 1999). The segments were cleared with 10% KOH and stained with 5% black ink-vinegar solution (Vierheilig et al. 1998). The roots were mounted on microscope slides in poly-vinyl-lacto glycerol (Koske and Tessier 1983) and observed under the compound microscope to determine the percentage of mycorrhizal root segments (Brundrett et al. 1996). Total colonization was assessed by averaging the percentages of ectomycorrhizal formations and the presence of the mantle in each root segment.

2.2.4 NaCl and fluoride treatments

One month after fungal inoculation, the seedlings of each plant species were divided into six groups, each with 16 seedlings. Each group was treated with one of the following solutions: distilled water (control), 60 mM NaCl, 1mM KF, 5mM KF, 60mM NaCl + 1mM KF, and 60mM NaCl + 5mM KF. The treatments were applied every third day for three weeks by immersing the pots in treatment solutions for 24 h and draining them for the next 48 h. Modified Hoagland's mineral solution was applied once a week with treatment solutions. After every third treatment application, the soil was flushed with de-ionized water to minimize salt build-up.

2.2.5 Transpiration rates and needle necrosis

Transpiration rates (E) were measured after four weeks of treatments using a steady state porometer LI-600 (LI-COR Biosciences, Linconln, NE, USA) in the uppermost branches of eight seedlings (n = 8) per treatment combination per plant species. The measurements started four hours following the onset of the photoperiod. Needle surface areas were calculated with Sigma Scan 5.0 scanning software (Jandel Scientific, San Rafael, CA, USA).

Needle necrosis was estimated in eight seedlings (n = 8) per treatment combination per plant species. Each seedling was inspected for the percentage of needles with necrosis (a) and for the percentage of needle areas affected by necrosis (b). Needle necrosis index (I) was calculated from the following equation: $I = a * b/1000$ Therefore, the range was from 0 (no needle necrosis) to 10 (all needles with 100% necrosis).

2.2.6 Seedling dry weights and root hydraulic conductance (K_r)

After three weeks of treatments, eight seedlings per treatment combination per plant species (n = 8) were harvested. Roots were excised and washed with distilled water. Shoot and root dry weights were determined after freeze-drying for 48 hours.

Root hydraulic conductance (K_r) was measured with a high pressure flow meter (HPFM, Dynamax Inc., Houston, TX, USA). The shoots of six seedlings for each plant species (n = 6) from the control, 1mM KF, 60mM NaCl and 1mM KF + 60mM NaCl treatments were excised about 1.5 cm above the root collar and the roots attached through the cut stem to the HPFM. The roots were pressurized to 0.5MPa to obtain a pressure-flow relationship for the calculations of K_r (Voicu et al. 2008).

2.2.7 Tissue analysis of Cl and F

Root and shoot Cl and F concentrations were analyzed in six seedlings (n = 6) per treatment combination per plant species. For the Cl determinations, roots and shoots were freeze-dried for 48 h and pulverized with a Willey mill. Tissue samples (50 mg) were placed in test tubes and 6 ml deionized water was added to each tube. The tubes were immersed in a hot water bath at 90°C for 10 min, placed on an orbital shaker for another 20 min and then centrifuged at 3500 rpm for 10 min. The centrifuged extracts were decanted, 6 ml of deionized water was again added and the whole process was repeated. The extracts were combined and filtered through a 0.45 μm Millipore filter before the analysis. Chloride was analyzed using a DI 300 ion chromatograph (Dionex; Sunnyvale, CA). For the F determinations, freeze-dried samples (250 mg) were extracted using the alkali fusion method (McQuaker and Gurney 1977). Fluoride concentrations in tissue extracts were determined with a fluoride selective electrode (Fisher Scientific, Toronto, ON, Canada).

2.2.8 Experimental design and statistical analysis

The experiment had a nested design with a 3 x 2 factorial test structure for each of the tree species used. There were two NaCl levels (0 and 60mM), and three KF levels (0, 1, and 5mM). The analysis of variance was performed using the Mixed Procedure of SAS (Version 9.1, SAS Institute Inc.; Cary, NC, USA). The data for K_r in black spruce seedlings and Cl in black spruce, white spruce and jack pine seedlings were transformed by the $\log_{10}$ function. The data for leaf F concentration in black spruce seedlings were transformed by the 'sqrt' function. In both cases, the transformations were done in order to meet the ANOVA assumptions of normality of distribution and homogeneity of variance. The transformed means and their standard errors were back-transformed for presenting in figures. Comparisons were used at $\alpha = 0.05$ for NaCl* KF interaction treatment means for each tree species. The comparisons were based on the results of the pdiff option from the Proc Mixed procedure from SAS (Littell et al. 2006). ANOVA p-values for the main factors and their interactions are reported in Table 2.1.

The following experimental model was used for each tree species:

$Y_{ijkl} = \mu + N_i + F_j + N_iF_j + T_k(N{*}F) + e_{ijkl}$, where

Y_{ijk} = value of individual observation (*i*= NaCl, *j*= KF, *k*= observation)

μ = overall mean of observations

N_i = effect of i^{th} treatment (i = NaCl treatment)

F_j = effect of j^{th} treatment (j = KF treatment)

N_iF_j = interaction effect of i^{th} and j^{th} treatments

$T_k(N{*}F)$ = error term (k= tray number)

e_{ijkl} = residual error

2.3 Results

2.3.1 Root colonization

Mean percent colonization of inoculated, non-treated (control), plants was approximately 41% in white spruce and black spruce and 31% in jack pine (Table 2.2). The effects of KF and NaCl treatments on root colonization varied with plant species and applied treatment. Fungal colonization of white spruce and jack pine roots subjected to 5 mM KF or 5 mM KF + 60 mM NaCl treatment was significantly greater compared with the 60 mM NaCl treatment (Table 2.2)

2.3.2 Plant dry weights and needle necrosis

NaCl and KF treatments reduced total dry weights in jack pine and black spruce seedlings, while they did not affect total dry weights in white spruce seedlings (Figure 2.1a). The effect of NaCl and KF on dry weights in both jack pine and black spruce was additive and in seedlings treated with 1 mM KF + 60 mM NaCl dry weights were reduced by about 30% in black spruce ($p < 0.001$) and 40% in jack pine ($p = 0.002$) compared with the control (Figure 2.1a). Shoot to dry weight root ratios were largely unaffected by the NaCl and KF treatments except for the 1 mM KF + 60 mM NaCl which increased shoot to root ratios in black spruce by about 30% compared with control seedlings ($p = 0.008$) (Figure 2.1b).

Treatment with 60 mM NaCl did not have a significant effect on the needle necrosis index in any of the three studied plant species (Figure 2.1c). Treatments with KF and KF + NaCl produced different extents of needle injury in seedlings in different plant species. In black spruce, 1 mM and 5 mM KF resulted in the needle necrosis index values of about 3.7 and 6.0, respectively (Figure 2.1c). However, in the presence of 60 mM NaCl, the needle necrosis indices were reduced to approximately 1.4 and 1.8 for the 1 mM KF + 60 mM NaCl and 5 mM KF + 60 mM KF treatments, respectively (Figure 2.1c). In white spruce, KF treatments produced less needle injury compared with black spruce and only the higher, 5 mM KF concentration significantly increased the needle necrosis index (Figure 2.1c). Similarly to black spruce, 60 mM NaCl + KF treatments produced less needle injury compared with KF alone (Figure 2.1c). Needle injury index in jack pine increased to about 2.0 and 3.4 in seedlings treated with 1 mM and 5 mM KF, respectively. Contrary to black spruce and white spruce, these values in jack pine seedlings were not significantly affected by the exposure to 60 mM NaCl (Figure 2.1c).

2.3.3 Transpiration rates (E) and root hydraulic conductance (K_r)

In all three tree species, transpiration rates (E) were markedly reduced by the 60 mM NaCl treatment, compared with untreated controls (Figure 2.2a). Both 1 mM and 5 mM KF also reduced E values, but the reductions were less profound than those induced by NaCl, particularly in white spruce and jack pine seedlings (Figure 2.2a). The addition of 1 mM and 5 mM KF to the 60 mM NaCl solutions did not significantly affect E responses to NaCl (Figure 2.2a).

Root hydraulic conductance (K_r) was examined in black spruce, white spruce and jack pine seedlings treated with 1 mM KF, 60 mM NaCl, 1 mM KF + 60 mM NaCl, and non-treated control. In black spruce, K_r significantly decreased in seedlings treated with 60 mM NaCl and 1 mM KF + 60 mM NaCl (Figure 2.2b). In white spruce, there was an increase in K_r in seedlings treated with 1 mM KF that was significantly different from 60 mM NaCl and 1 mM KF + 60 mM NaCl treatments (Figure 2.2b). There was no effect of any of the NaCl and KF treatments of K_r in jack pine (Figure 2.2b).

2.3.4 Tissue concentrations of Cl and F

In all treatments containing NaCl (60 mm NaCl, 1 mM KF + 60 mM NaCl, and 5 mM KF + 60 mM NaCl), seedlings accumulated more Cl in the shoots compared with roots (Figure 2.3a,b,c). However, for all NaCl treatments, the shoot to root ratios and shoot concentrations of Cl in white spruce were lower compared with black spruce and jack pine (Figure 2.3a,b,c). Root and shoot Cl concentrations in plants of all three examined plant species were not affected by the presence of 1 mM and 5 mM KF in 60 mM NaCl treatments (Figure 2.3a,b,c).

Seedlings treated with 1 mM KF accumulated between about 7 and 12 µg F g^{-1} DW depending on the plant species and these concentrations were not significantly affected by the presence of 60 mM NaCl (Figure 2.4). When treated with 5 mM KF, black spruce shoots accumulated approximately 54 µg F g^{-1} DW and this concentration was reduced to about one-half by the presence of 60 mM NaCl in treatment solution (Figure 2.4). Similarly, in white spruce, shoot fluoride concentrations of about 26 µg F g^{-1} DW that were measured in plants treated with 5 mM KF were reduced to below 13 µg F g^{-1} DW in 5 mM KF + 60 mM NaCl treatment (Figure 2.4). In jack pine, mean shoot F concentrations were 30.1 and 24.5 µg F g^{-1} DW in seedlings treated with 5 mM KF and 5 mM KF + 60 mM NaCl, respectively (Figure 2.4), and the difference was not significant at $\alpha = 0.05$.

2.4 Discussion

Treatments with KF and KF + 60 mM NaCl reduced plant dry weights in ECM black spruce and jack pine, but not in white spruce seedlings inoculated with *Suillus tomentosus* (Figure 2.1a). In an earlier study (Nguyen et al. 2006), I demonstrated that NaCl treatment reduced plant dry weights in ECM white spruce and black spruce inoculated with *Hebeloma crustuliniforme* and *Laccaria bicolor*, but did not affect dry weights in ECM jack pine seedlings. These differences in growth responses between the plant species and between the present and earlier (Nguyen et al. 2006) studies is likely due to differences in compatibilities between the different ECM fungal species and their plant hosts (Smith and Read 1997, Dahlberg and Finlay 1999). Clearly, more studies are required to understand the interactions between different mycorrhizal associations since root colonization values alone may not reflect quantitative differences in hyphal development and hyphal root penetration which may affect plant physiological responses (Repáč 2008).

Although when treated separately, KF and 60 mM NaCl treatments reduced E values compared with non-treated control seedlings, the reduction of E by 60 mM NaCl was not aggravated by the presence of 1 mM or 5 mM KF in treatment solutions (Figure 2.2a). Leaf stomatal conductance and transpiration rates were found to be very sensitive in plants exposed to gaseous (Rakowski and Zwiazek 1992) and soil (Kamaluddin and Zwiazek 2003) fluoride. However, to the best of our knowledge, the effects of fluoride had not been previously studied in ECM plants. Therefore, it is possible that similarly to NaCl (Muhsin and Zwiazek 2002, Langenfeld-Heyser et al. 2007, Calvo Polanco et al. 2008) and other soil pollutants

(Jones and Hutchinson 1988, Jentschke and Godbold 2000), ECM associations modulated the physiological responses of plants to KF.

Treatment solutions containing 60 mM NaCl reduced root hydraulic conductance (K_r) in black spruce, but not in white spruce and jack pine (Figure 2.2b). Fluoride and NaCl are strong inhibitors of the aquaporin-mediated water transport in roots (Kamaluddin and Zwiazek 2003, Martínez-Ballesta et al. 2006). Therefore, it is possible that the differences in K_r responses to NaCl may reflect differences in relative contributions of the aquaporin-mediated water transport in the three examined conifer species. In some plants, ECM associations strongly enhance the aquaporin-mediated root water transport (Marjanović et al. 2005), which is also a likely significant factor contributing to the responses of K_r to KF and NaCl in ECM plants.

Treatments with 1 mM and 5 mM KF produced significant needle injury in the three studied conifer species (Figure 2.1c). However, for the 1 mM and 5 mM KF treatments, the needle necrosis index was higher in black spruce and jack pine compared with white spruce. It is interesting that in black spruce and white spruce the needle necrosis index was lower in the plants treated with KF + 60 mM NaCl solutions compared with those containing only KF (Figure 2.1c). In black spruce and white spruce, the reduction in needle necrosis in plants treated with KF + 60 mM NaCl compared with KF treatments coincided with a reduction in transpiration rates (Figure 2.2a). Since fluoride is transported with the transpiration stream (Heath 1980, Weinstein and Davison 2004), changes in transpiration rates could potentially affect its rate of uptake from the soil. A similar correlation has been reported for the uptake of Na and Cl (Munns and Termaat 1986, Dalton et al. 2000). However, in addition to the volume of the transpiration flux, the rate of salt accumulation in the leaves depends mainly on the ability of the roots to exclude salt from the transpiration stream (Munns and Termaat 1986). In jack pine, the transport of Na ions to the shoot was related to the presence of Cl, but not to the transpiration rate (Franklin and Zwiazek 2004). It has been suggested that Cl is the main factor contributing to the loss of membrane integrity and reduced capacity of roots for Na sequestration in the roots of NaCl-treated plants (Bernstein 1975, Redfield and Zwiazek 2002, Franklin and Zwiazek 2004).

In the present study, Cl concentrations were higher in the shoots compared with roots in the three examined plant species (Figure 2.3a,b,c). Many plant species are not able to effectively regulate Cl entry into the shoot and accumulate Cl in greater amounts than Na (Jacoby 1994). In jack pine, Cl uptake and transport were independent of external concentration, suggesting that rates are limited by the number of ion channels or carriers (Franklin and Zwiazek 2004). This agrees with the generally accepted model of Cl transport across the plasma membrane as an active process (Maas and Ogata 1972, Tyerman and Skerrett 1999).

In our study, KF had no significant effect on the distribution and concentration of Cl in ECM plants. Similarly, it was reported for barley that F had little effect on Cl uptake (Elzam and Epstein 1965). On the other hand I have found F shoot concentrations to be reduced in white spruce and black spruce seedlings by the presence of NaCl in the 5 mM KF treatment solutions (Figure 2.4a,b) and this reduction correlated with the decrease in needle necrosis in both spruce species (Figure 2.1c). Although the exact pathways of F transport are not well understood, it is possible that, similarly to NO_3^- (White and Broadley 2001), Cl may also share membrane channels with F. There are several types of Cl-permeable channels that may be present in the plasma membranes and tonoplasts of plants that may have a higher specificity for F than Cl (Skerrett and Tyerman 1994, White and Broadley 2001). Although plant Cl channels have been well characterized, little is known about the transport of Cl across hyphal membranes in mycorrhizal fungi.

The results of our study suggest a possible competitive inhibition of F transport by Cl. However, it has been suggested that F is transported across cell membranes partly in an uncharged form of HF (Kronberger 1988). It could be speculated that, similarly to many other small uncharged molecules (Maurel 2007), transmembrane transport of fluoride may also involve aquaporins. The amount of boron transported through the plasma membrane aquaporins was significant at high external B concentrations (Dordas and Brown 2001, Bastías et al. 2004). Since, the activity of aquaporins is strongly inhibited by NaCl (López-Berenguer et al. 2006, Martínez-Ballesta et al. 2006), this could help explain the reduction of F tissue concentrations in white spruce and black spruce seedlings treated with 5 mM KF + 60 mM NaCl, but not with 1 mM KF + 60 mM NaCl. However, this hypothesis will require further studies. The lack of effect of NaCl on F shoot concentrations in jack pine suggests that the transport mechanisms of F may vary between plant species or that these properties were differently affected by *S. tomentosus* in jack pine compared with the studied spruce species.

In conclusion, I showed that the presence of 60 mM NaCl alleviated fluoride-induced needle injury in ECM black spruce and white spruce seedlings, but had little effect in jack pine seedlings. Both KF and 60 mM NaCl treatments reduced E values compared with non-treated control seedlings. However, with the exception of small reductions of K_r by NaCl treatments in black spruce, the applied KF and NaCl treatments had little effect on K_r in ECM plants. Chloride tissue concentrations in NaCl-treated plants were not affected by the presence of KF in treatment solutions. However, shoot F concentrations in ECM black spruce and white spruce treated with 5 mM KF + 60 mM NaCl were significantly reduced compared with the 5 mM KF treatment. The results point to a possible competitive inhibition of F transport by Cl. I also suggest that the possibility that aquaporins may be involved in the transmembrane transport of F should be further investigated.

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Table 2.1. ANOVA p-values for the main factors and their interactions for each ECM tree species. The main factors are: 0, 1, and 5 mM KF and 0 and 60 mM NaCl treatments. The measured parameters includes total dry weights (DW), shoot to root ratios, leaf necrosis index, transpiration (E), root hydraulic conductance (K_r), root and shoot Cl, and shoot F concentrations.

	White spruce			Jack pine			Black spruce		
	KF	NaCl	KF*NaCl	KF	NaCl	KF*NaCl	KF	NaCl	KF*NaCl
Total DW	0.033	0.698	0.990	0.009	0.127	0.780	<0.001	0.004	0.993
Shoot:root ratio	0.866	0.984	0.682	0.821	0.645	0.941	0.002	0.120	0.400
E	0.179	<0.001	0.141	0.021	<0.001	0.236	<0.001	<0.001	<0.001
Necrosis	<0.001	<0.001	0.001	<0.001	0.047	0.952	<0.001	<0.001	<0.001
K_r	0.051	0.034	0.100	0.567	0.972	0.658	0.107	0.002	0.951
Cl Roots	0.642	<0.001	0.001	0.115	<0.001	0.039	0.003	<0.001	0.002
Shoots	0.657	<0.001	0.656	0.509	<0.001	0.007	0.550	<0.001	0.929
F	<0.001	<0.001	<0.001	<0.001	0.539	0.261	<0.001	0.801	0.201

Table 2.2. Percentages of root lengths colonized with mycorrhizal fungi in black spruce, jack pine and white spruce seedlings inoculated with *Suillus tomentosus* and subjected to 0 or 60 mM NaCl, 0, 1 or 5 mM KF or their combinations for 25 days. The values are means ± SE of 6 seedlings per treatment (n=6). Different letters within the same column (the same tree species) indicate significant differences at α = 0.05.

	Black spruce	White spruce	Jack pine
Non-treated control	40.8 ± 12.1ab	40.8 ± 4.2abc	31.0 ± 5.4ab
60 mM NaCl	52.9 ± 14.0ab	22.3 ± 2.9a	17.4 ± 2.7a
1 mM KF	62.5 ± 11.2a	40.6 ± 7.1abc	28.0 ± 3.8a
1 mM KF + 60mM NaCl	48.8 ± 5.4ab	28.9 ± 5.6ab	26.0 ± 5.3a
5 mM KF	30.9 ± 3.9b	46.2 ± 3.6c	46.8 ± 7.1b
5 mM KF + 60mM NaCl	46.4 ± 8.9ab	36.5 ± 4.1bc	46.8 ± 10.8b

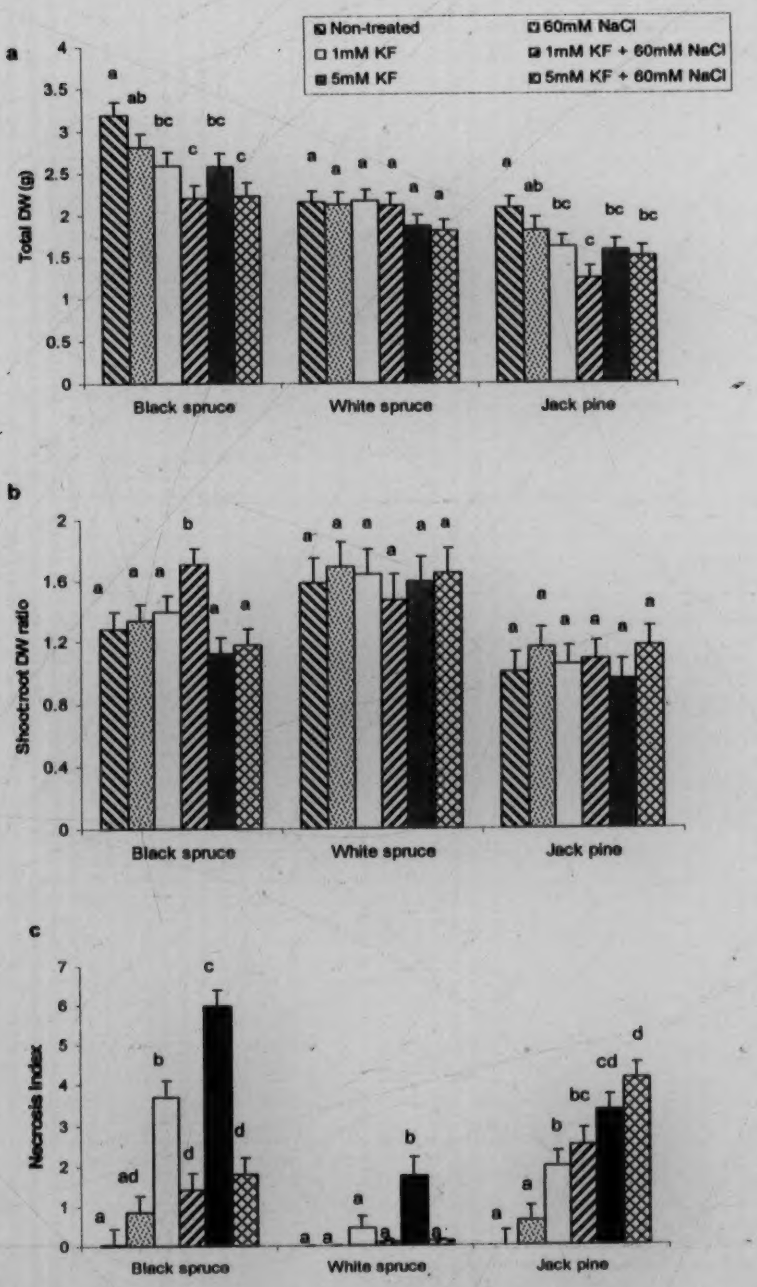

Figure 2.1. Total dry weights (a) shoot:root ratios (b) and needle necrosis index (c) in black spruce, white spruce and jack pine seedlings. The seedlings were inoculated with *Suillus tomentosus* and treated with 60 mM NaCl, 1 mM KF, 5 mM KF, 1 mM KF + 60 mM NaCl or 5 mM KF + 60 mM NaCl for three weeks. For each tree species, different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments. Means ($n = 8$) + SE are shown.

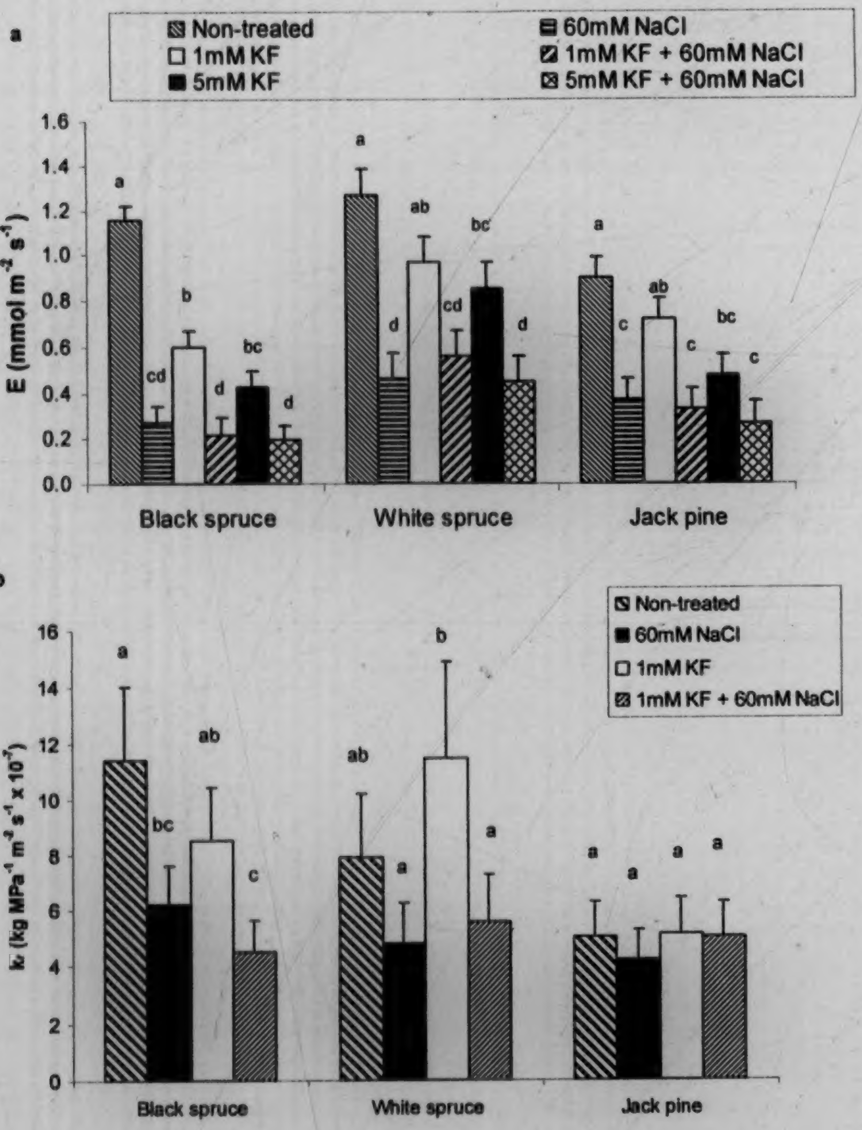

Figure 2.2. Transpiration rates (E) (a) and root hydraulic conductance (K_r) (b) in black spruce, white spruce and jack pine seedlings. The seedlings were inoculated with *Suillus tomentosus* and treated with 60 mM NaCl, 1 mM KF, 5 mM KF, 1 mM KF + 60 mM NaCl or 5 mM KF + 60 mM NaCl for three weeks. For each tree species, different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments. Means (n = 7) + SE are shown.

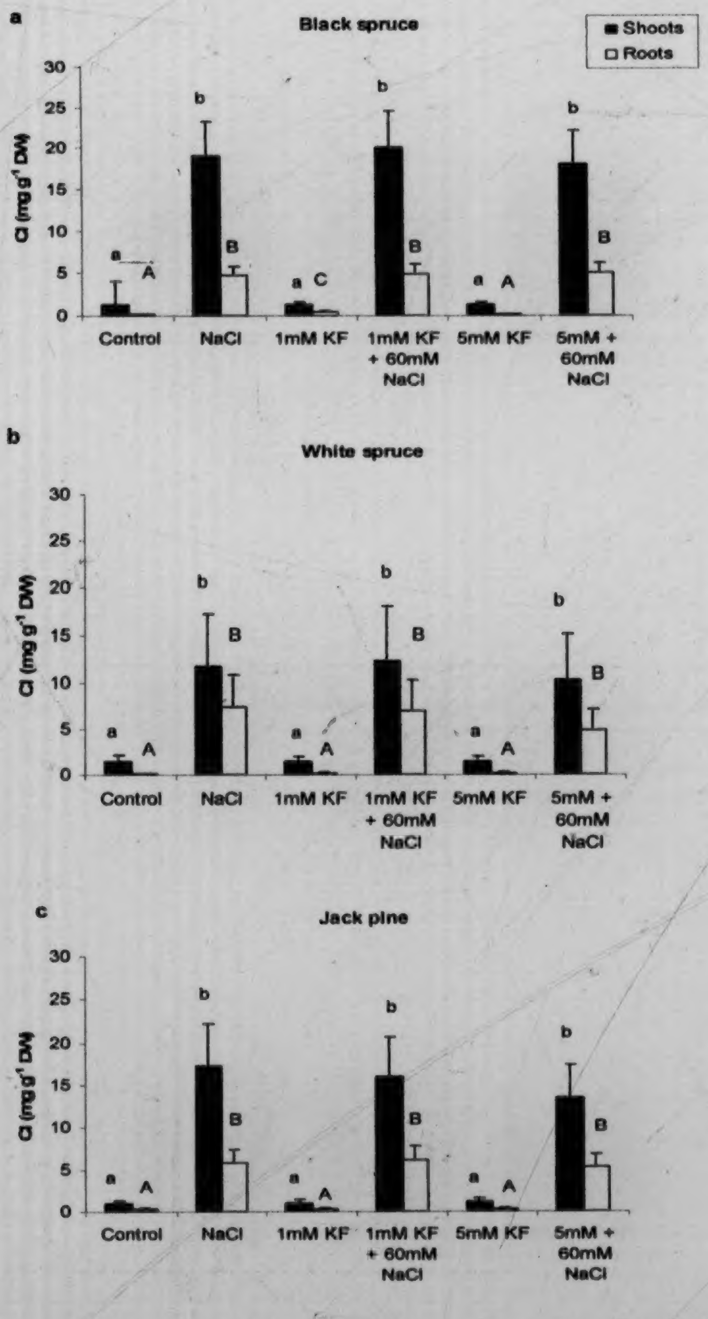

Figure 2.3. Concentrations of Cl in roots and shoots of black spruce (a), white spruce (b) and jack pine (c) seedlings. The seedlings were inoculated with *Suillus tomentosus* and treated with 60 mM NaCl, 1 mM KF, 5 mM KF, 1 mM KF + 60 mM NaCl or 5 mM KF + 60 mM NaCl for three weeks. Lowercase and uppercase letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments in shoots and roots, respectively. Means ($n = 6$) + SE are shown.

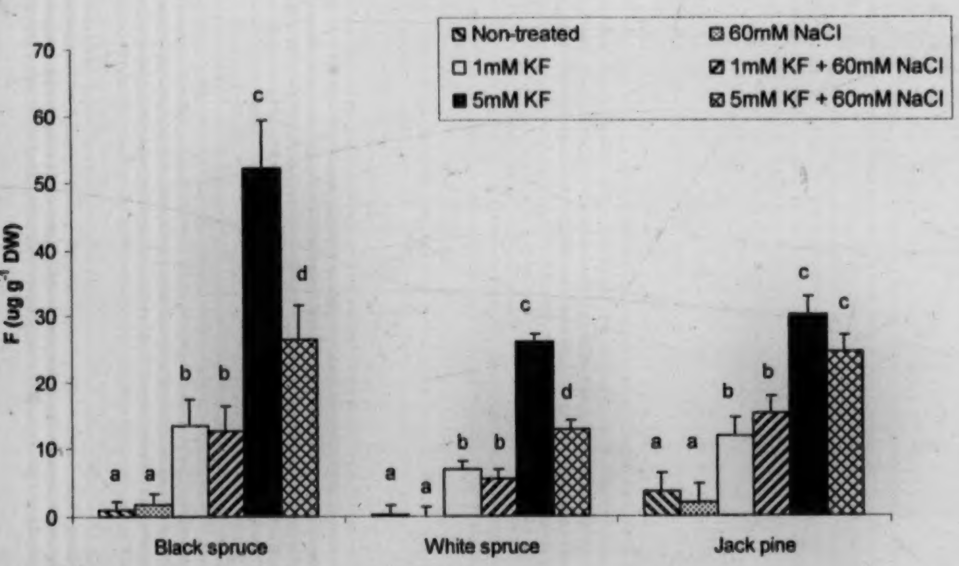

Figure 2.4. Concentrations of F in shoots of black spruce, white spruce and jack pine seedlings. The seedlings were inoculated with *Suillus tomentosus* and treated with 60 mM NaCl, 1 mM KF, 5 mM KF, 1 mM KF + 60 mM NaCl or 5 mM KF + 60 mM NaCl for three weeks. For each tree species, different letters above the bars indicate significant differences ($\alpha = 0.05$) between treatments. Means ($n = 6$) + SE are shown.

CHAPTER III*: Responses of mycorrhizal jack pine (*Pinus banksiana*) seedlings to NaCl and boron

3.1 Introduction

Boron is an essential micronutrient involved in maintaining cell wall structure and function, enzyme activation, nucleic metabolism and carbohydrate transport (Loomis and Durs, 1992, Power and Woods 1997). However, when present in high concentrations, B affects plants by reducing leaf chlorophyll contents, inhibiting photosynthesis, retarding cell wall formation (Lovatt and Bates 1984, Nable et al. 1997), and inhibiting root growth (Apostol et al. 2002). Elevated B levels are often present in saline soils, particularly in areas with poor drainage or with shallow water tables that limit opportunities for leaching (Gupta et al. 1985, Renault et al. 1998). Salts and B have been identified among the main soil factors affecting plant growth and survival in the vast areas reclaimed following oil sands mining in northeastern Alberta, Canada. It has been reported that oil sands tailings waters contain as much as 4 mg L^{-1} B (Renault et al. 1999). This is largely due to the presence of process-affected waters which are produced during the bitumen extraction using hot-water caustic processes and which contain elevated concentrations of salts and B (FTFC 1995).

Plants transport B with the transpiration stream to the leaves where chlorotic and necrotic lesions often appear at the tips and margins (Brown and Shelp 1997,

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Apostol and Zwiazek 2004). Boron may aggravate salt injury and enhance Na concentration in the shoots (Apostol et al. 2002). Similarly to NaCl, the ability of plants to tolerate elevated soil B levels largely depends on the restriction of uptake and sequestration of B in less sensitive parts of the plant (Nable et al. 1997). In *Pinus banksiana* (Apostol et al. 2002), *Eucalyptus* sp. (Marcar et al. 1999) and *Zea mays* (Bastías et al. 2004), B uptake was reduced by NaCl treatment. Although the exact mechanisms of B uptake by plant roots are not fully understood, aquaporins have been implicated in B transport across cell membranes (Dordas and Brown 2001, Bastías et al. 2004). Since NaCl strongly inhibits aquaporin-mediated transport (Martínez-Ballesta et al. 2006) it is possible that aquaporins may be involved in the observed reduction in B uptake by plants treated with NaCl.

It has been well established that tree responses to adverse environmental conditions are affected by the presence of mycorrhizal associations. These associations supply trees with essential nutrients (Marschner and Dell 1994, Smith and Read 1997) and enhance water uptake (Landhäusser et al. 2002, Muhsin and Zwiazek 2002a,b). Mycorrhizas can alleviate metal toxicity (Jones and Hutchinson 1988, Jentschke and Godbold 2000) likely through the sorption of metals to fungal tissues and intracellular uptake and detoxification in fungal vacuoles (Denny and Wilkins 1987, Hartley et al. 1997). I have also demonstrated (Muhsin and Zwiazek 2002a, Nguyen et al. 2006) that mycorrhizal associations alleviate NaCl injury in plants by reducing shoot Na concentrations.

Little is known about B transport in mycorrhizal plants. Although B is not considered to be an essential element to fungi, Lehto et al. (2004) demonstrated that mycorrhizal mycelium of *Paxillus involutus* absorbed and transported B to the host birch tree. Since mycorrhizal formation increases aquaporin-mediated transport by increasing transcript levels of root aquaporins (Marjanović et al. 2005), mycorrhizas could also potentially affect B uptake by plant roots.

In the present study, I examined the effects of B on NaCl resistance in jack pine (*Pinus banksiana* Lamb.) seedlings inoculated with *Hebeloma* sp., *Suillus tomentosus* (Kauffman) Singer, Snell and Dick, and *Wilcoxina mikolae* var. *mikolae* (Yang and Wilcox) Yang and Korf. Both *Hebeloma* sp. (Bois et al. 2006) and *Suillus tomentosus* (Danielson 1984, Danielson and Visser 1989) are ectomycorrhizal (ECM) with jack pine. *Wilcoxina* spp. form ectendomycorrhizal associations with pines (Scales and Peterson 2004, Wagg et al. 2008). These associations are characterized by the presence of a mantle and Hartig net but they differ from ECM in that intracellular hyphae develop in the epidermal and cortical cells (Peterson et al. 2004). I hypothesized that the presence of fungal mycelia would increase B uptake by the host plants in the presence and absence of NaCl.

3.2 Materials and Methods

3.2.1 Plant material and growth conditions

Jack pine (*Pinus banksiana*) seeds were obtained from cones collected in forest stands near Fort McMurray, AB, Canada. The seeds were surface-sterilized with 5% H_2O_2 for one minute and germinated in Petri dishes on moist sterile sand. One week after germination, the seedlings were transplanted to 170 ml Spencer-Lemaire root trainers

(Spencer-Lemaire Industries Ltd. Edmonton, AB, Canada) filled with a mixture of peat moss and sand (2:1 v/v). The soil mixture was sterilized by autoclaving 2 x 20 min on two different days at 121°C. The seedlings were placed in a growth room set to 22/18°C day/night temperature, 16-h photoperiod, 65 ± 10% relative humidity, and 350 μmol m^{-2} s^{-1} photosynthetic photon flux density at the seedling level. Modified Hoagland's mineral nutrient solution (Epstein and Bloom 2005) was applied to the seedlings every fourth day. The soil was flushed weekly with de-ionized water to prevent ion accumulation. The concentration of mineral nutrients was lowered to 25% two weeks before inoculation to improve the success of fungal colonization.

3.2.2 Fungal inoculation

The seedlings were randomly divided into four groups of forty-eight seedlings. One group served as non-inoculated control and each of the other three groups was inoculated with one of the following fungal cultures obtained from the University of Alberta Microfungus Collection and Herbarium (UAMH): *Wilcoxina mikolae* var. *mikolae* (UAMH 6703), *Hebeloma* sp. (UAMH 9053), or *Suillus tomentosus* (UAMH 5506). All three fungal species were isolated from roots or sporocarps in northeastern Alberta (Canada). Modified Melin-Norkans (MMN) liquid medium (Mason 1980) was used to grow fungal cultures in 4 cm (diameter) x 60 cm custom-made glass cylindrical flasks aerated with a pump connected to the side arm of the glass flask through the PVC tubing. The fungal cultures were growing for four week in 1.5 l of liquid medium per flask. Twenty milliliters of the fungal mixture was applied to the surface of the root plug of four-month-old seedlings with a pipette after opening the root trainers.

3.2.3 Root fungal colonization

Six seedlings per treatment combination (n = 6) were randomly taken at the time of physiological measurements to determine fungal colonization. Six distal root segments (1-2-cm long) were removed from each root system and placed in the formalin-acetic acid-alcohol (FFA) fixative (Ruzin 1999). The root segments were cleared with 10% KOH and later stained with 5% black ink-vinegar solution (Vierheilig et al. 1998). The roots were mounted on microscope slides in poly-vinyl-lacto glycerol (Koske and Tessier 1983) and observed under the microscope to measure the percentage of root segments colonized by mycorrhizal fungi (Brundrett et al. 1996). Total colonization was assessed by averaging the percentage of ectomycorrhizal formations and the presence of the mantle in each root segment.

3.2.4 NaCl and boron treatments

The treatments started four weeks after inoculation by applying distilled water (control treatment), 60 mM NaCl, 2mM H_3BO_3 or 60mM NaCl + 2mM H_3BO_3. Twelve seedlings per fungal treatment were selected for each of the NaCl and boron treatments. One set of seedlings was left as non-treated control and received only distilled water or nutrient solution. The treatment solutions were applied every second day for four weeks. Once a week, modified Hoagland's mineral nutrient solution was applied with treatment solutions (Epstein and Bloom 2005). Once a week, the soil was flushed with distilled water to minimize salt build-up.

3.2.5 Gas exchange

After four weeks of treatments, transpiration rates (E) were measured in the uppermost branches of eight seedlings (n = 8) per treatment combination using a steady state porometer LI-600 (LI-COR Biosciences, Linconln, NE, USA) starting at four hours following the onset of the photoperiod. Needle surface areas were calculated with Sigma Scan 5.0 scanning software (Jandel Scientific, San Rafael, CA, USA).

3.2.6 Needle necrosis and chlorophyll concentrations

Chlorophyll was extracted from 20 mg freeze-dried needles (six seedlings per treatment combination, n = 6) with 8 ml methanol and determined spectrophotometrically (Ultrospec III, Pharmacia LKB, Uppsala, Sweden). Total chlorophyll concentrations were calculated using the MacKinney's equation (Šesták et al. 1971).

Needle necrosis was estimated in eight seedlings (n = 8) per treatment combination. Each seedling was assessed for the percentage of needles with necrosis (a) and for the percentage of total needle areas affected by necrosis (b). Needle necrosis index (I) was calculated from the following equation: $I = a * b/1000$. Therefore, the range was from 0 (no needle necrosis) to 10 (all needles with 100% necrosis).

3.2.7 Dry weights and tissue elemental analysis

Eight seedlings per treatment combination (n = 8) were harvested after four weeks of treatment. Roots of each seedling were excised and washed with distilled water. Dry weights were determined in roots and shoots after freeze-drying for 48 h.

Root and shoot Na, Cl, K, and B contents were analyzed in six seedlings (n = 6) per treatment combination. For the Cl determinations, tissue samples were freeze-dried for 48 hours and extracted with hot water. Ground tissue samples (50 mg) were placed in test tubes and 6 ml deionized water was added to each tube. The tubes were immersed in a hot water bath at 90°C for 10 minutes, placed on an orbital shaker for another 20 minutes and then centrifuged at 3500 rpm for 10 minutes. The extracts were then decanted and 6 ml of deionized water was again added and the whole process repeated. The extracts were combined and filtered through a 0.45 μm Millipore filter before the ion analysis. Chloride was analyzed using a DI 300 ion chromatograph (Dionex; Sunnyvale, CA). For the determination of Na, B and K, tissue samples were extracted with a wet ashing-nitric acid digestion method. Ground tissue samples (0.5-1 g) were extracted with 10 ml of HNO_3 and heated on a digestion block for 1 h. After cooling, the extracts were diluted to 100 ml with deionized H_2O and 20ml of the diluted extract was filtered through a 0.45 μm Millipore filter. The extracts were then analyzed using an inductively coupled plasma optical emission spectrometer (ICP-OES) (Vista-RL CCD Simultaneous ICP-OES, Varian Inc., Victoria, Australia).

3.2.8 Experimental design and statistical analysis

The experiment was a 4x2x2 complete randomized factorial design with three fungal species and a non-inoculated control, two NaCl levels (60 mM NaCl and 0 mM NaCl), and two B levels (2mM H_3BO_3 and 0 mM H_3BO_3). The analysis of variance was performed using the Mixed Procedure of SAS (Version 9.1, SAS Institute Inc.; Cary, NC, USA).. Data for the necrosis index was transformed with a 'sqrt' function to meet ANOVA assumptions of normality of distribution and homogeneity of variance. The values for the transformed means and their standard errors were back-transformed to present in the figures. The SLICE option from SAS (Littell et al. 2006) was used to examine the effects of the inoculation treatment (Non-Inocualted, *Hebeloma sp.*, *S. tomentosus* and *W.mikolae*) within the NaCl*B treatments and the effects of the NaCl*B treatments at each inoculation level (Table 3.1). Tukey's adjustment was used for paired-comparisons among treatment means at $\alpha=0.05$. ANOVA p-values for the main factors, their interactions, and for within and across inoculation treatments for the different compaction and salt interaction combinations are shown in Table 3.1.

The following experimental model was used:

$$Y_{ijkl} = \mu + M_i + N_j + B_k + M_iN_j + M_iB_k + N_jB_k + M_iN_jB_k + e_{ijkl}, \text{ where}$$

Y_{ijkl} = value of individual observation (*i*=mycorrhiza, *j*=NaCl, *k*=boron *l*=observation)

μ = overall mean of observations

M_i = effect of i^{th} treatment (i = mycorrhizal treatment)

N_j = effect of j^{th} treatment (j = NaCl treatment)

B_k = effect of k^{th} treatment (k= boron treatment)

M_iN_j = interaction effect of i^{th} and j^{th} treatments

M_iB_k = interaction effect of i^{th} and k^{th} treatments

N_jB_k= interaction effect of j^{th} and k^{th} treatments

$M_iN_jB_k$= interaction effect of i^{th}, j^{th} and k^{th} treatments

e_{ijkl} = residual error

3.3 Results

3.3.1 Root fungal colonization

Root colonization of control (untreated) seedlings was higher in plants inoculated with *Hebeloma* sp. and *W. mikolae* (~73-75% length of distal roots) compared with those inoculated with *S. tomentosus* (~54%) (Table 3.2). In non-inoculated seedlings, about 25% of the distal root lengths were found to be colonized by unidentified mycorrhizal fungi. With NaCl treatments, root colonization was reduced in the non-inoculated seedlings and those inoculated with *Hebeloma* sp. and *W. mikolae*. However, NaCl treatment had little effect on root colonization in seedlings inoculated with *S. tomentosus* (Table 3.2).

3.3.2 Dry weights

Inoculated plants had higher total dry weights compared with non-inoculated seedlings for all treatment combinations ($p < 0.001$) (Figure 3.1a). Dry weights of seedlings inoculated with *Hebeloma* sp. were almost twofold greater compared with non-inoculated plants (Figure 3.1a). Treatment with 60 mM NaCl reduced total dry weights of *Hebeloma* sp. ($p = 0.013$) seedlings but did not significantly affect dry weights of seedlings inoculated with *S. tomentosus* ($p = 0.990$) and *W. mikolae* ($p = 0.998$).There was no effect of B on total dry weights ($p = 0.476$) (Figure 3.1a).

Shoot:root dry weight ratios increased as a result of mycorrhizal inoculation ($p < 0.001$) (Figure 3.1b). There was no effect of NaCl and B treatments on shoot:root ratios in inoculated and non-inoculated seedlings ($p = 0.858$) (Figure 3.1b).

3.3.3 Transpiration rates, needle chlorophyll concentrations and necrosis

Transpiration rates (E) were measured in non-inoculated seedlings and in the plants inoculated with *S. tomentosus*. For all treatments, there was no significant effect of mycorrhizal inoculation on E values (Figure 3.2a). Treatment with 60 mM NaCl and 2 mM H_3BO_3 + 60 mM NaCl reduced E by about fourfold, but a small reduction in E of 2 mM H_3BO_3 treatment was not significant at $\alpha = 0.05$ (Figure 3.2a).

Needle chlorophyll concentrations were higher in inoculated seedlings compared with the non-inoculated plants within the different treatment combinations (Figure 3.2b). Both B ($p < 0.001$) and NaCl ($p = 0.002$) treatments resulted in significant decreases in chlorophyll concentrations (Figure 3.2b).

0.086 and $p = 0.012$, respectively) (Figure 3.2c). There was no effect of 2 mM H_3BO_3 treatment on needle necrosis within experimental groups (Figure 3.2c) and there was no significant effect of inoculation treatment on needle necrosis ($p = 0.076$). However, when B was applied with NaCl, needle necrosis increased in all inoculation groups (Figure 3.2c).

3.3.4 Root and shoot concentrations of Na, Cl, B and K

Both root and shoot Na concentrations increased in plants treated with NaCl ($p < 0.001$) and NaCl + B ($p < 0.001$) (Figure 3.3a,b). The root Na concentrations were similar in all inoculation groups (Figure 3.3a). However, inoculated plants had lower shoot Na concentration compared with non-inoculated seedlings. In the 60 mM NaCl treatment, the lowest shoot Na concentration was present in the seedlings inoculated with *W. mikolae* ($p = 0.002$) (Figure 3.3b). In the 2 mM B + 60 mM NaCl treatment, B resulted in a significant decline of shoot Na concentrations in inoculated seedlings ($p = 0.003$) (Figure 3.3b).

There were only small differences in Cl root concentrations between inoculated and non-inoculated plants and between NaCl and B + NaCl treatments (Figure 3.4a). Shoots had several-fold higher Cl concentrations compared with roots (Figure 3.4a,b). In plants treated with 60 mM NaCl, the shoots of inoculated plants had lower Cl concentrations ($p < 0.001$) than those of non-inoculated seedlings (Figure 3.4b). However, an opposite trend was observed in NaCl + B treatment, which resulted in a higher accumulation of Cl in the shoots of inoculated plants ($p < 0.001$) (Figure 3.4b).

When treated with NaCl + B, the shoots of non-inoculated plants had only about 50% of the Cl concentration that was present in the NaCl treatment (Figure 3.4b).

The root concentration of K decreased by up to three-fold in plants treated with 60 mM NaCl and 2 mM B + 60 mM NaCl (Figure 3.5a). The opposite was observed in the shoots, where K concentrations increased by almost 50 % in response to both treatments containing NaCl (Figure 3.5b). Fungal inoculation had only minor effects on K shoot and root concentrations (Figure 3.5a,b).

Both treatments containing B (2 mM B and 2 mM B + 60 mM NaCl) resulted in an almost doubling of B concentration in roots (Figure 3.6a) and more than a 100% increase in the shoots (Figure 3.6b) of inoculated and non-inoculated plants. Fungal inoculation did not have a major effect on B root concentrations ($p = 0.146$) although small decreases in B shoot concentrations were observed ($p = 0.458$) in inoculated plants in some treatments (Figure 3.6a,b).

3.4 Discussion

Inoculation of jack pine seedlings with the three studied fungi increased dry weights and shoot:root ratios compared with the non-inoculated seedlings. These growth responses do not appear to be related to colonization rates (Table 3.2). Dry weights of control seedlings inoculated with *Hebeloma* sp. were almost two-fold higher than those of non-inoculated plants (Figure 3.1a). Although the majority of greenhouse studies showed growth stimulation (Nguyen et al. 2006, Karst et al. 2008), there is evidence of publication bias against experiments showing growth depressions in plants inoculated with mycorrhizal fungi (Karst et al 2008). Similarly, changes in the shoot:root dry weight ratios can vary between different plant species inoculated with the same mycorrhizal fungus (Nguyen et al. 2006).

Both 60 mM NaCl and 2 mM B + 60 mM NaCl treatments significantly reduced dry weights in plants inoculated with *Hebeloma* sp. bringing the dry weights down to a level similar to those of other inoculation groups (Figure 3.1a). Although growth reductions are commonly observed in NaCl-treated plants (Apostol et al. 2002, Franklin and Zwiazek 2004), NaCl concentrations as high as 60 mM have also been reported to have little or no effect on shoot and root dry weights in non-mycorrhizal and mycorrhizal *Pinus banksiana* (Bois et al. 2006) and *Picea glauca* (Muhsin and Zwiazek 2002a, Bois et al. 2006). Similarly to untreated plants, the effects of mycorrhizal associations on growth of plants treated with NaCl vary between plant species (Nguyen et al. 2006, Yi et al. 2008).

Unlike in the earlier studies (Apostol et al. 2002, Apostol and Zwiazek 2004), 2 mM B treatment produced very little needle necrosis (Figure 3.2c) and I observed no significant effects of B on transpiration rates in non-inoculated plants (Figure 3.2a). However, B significantly reduced needle chlorophyll concentrations in non-inoculated plants (Figure 3.2b). The observed differences in the responses of plants to B treatment between the present and earlier (Apostol et al. 2002, Apostol and Zwiazek 2004) studies may be due to lower B tissue concentrations that were present in shoots and roots of plants in the present study. Boron and NaCl have been suggested to have a cumulative effect on plants (Apostol et al. 2002). Since B is likely transported through aquaporins (Dordas and Brown 2001) and both NaCl and mycorrhizas affect the function and expression of aquaporins (Marjanović et al. 2005, Martínez-Ballesta et.

al. 2006), I hypothesized that both factors would affect B uptake by jack pine seedlings. However, in the present study, the differences in B shoot concentration between the 2 mM B and 2 mM B + 60 mM NaCl treatments were relatively small (Figure 3.6a,b). This is in contrast to the reductions in B concentrations that were reported for jack pine and other plant species treated with B and NaCl (Marcar et al. 1999, Apostol et al. 2002, Bastías et al. 2004). Similarly, I observed little effect of mycorrhizal fungi on B root and shoot concentrations suggesting that B uptake were likely determined by other factors than aquaporins.

Similarly to earlier studies (Muhsin and Zwiazek 2002a, Bois et al. 2006, Nguyen et al. 2006), mycorrhizal associations reduced shoot Na concentrations in jack pine seedlings (Figure 3.3b). Although the exact mechanisms of this response are not known, it may be partly due to Na sequestration by the fungal hyphae. It is interesting that *W. mikolae*, which forms ectendomycorrhizal associations with jack pine (Peterson et al. 2004), was as effective in reducing shoot Na concentrations as the ECM fungi *Hebeloma sp.* and *S. tomentosus*. *Wilcoxina* spp. are very common colonizers of young conifer seedlings in northern temperate forests and in nurseries (Hagerman et al. 1999, Mah et al. 2001, Izzo et al. 2006, Rudawska et al. 2006) and hence, this species has a strong potential for being used as an inoculant for young seedlings destined for sites high in Na.

When treated with NaCl + B, the shoots of non-inoculated plants had about 50% lower Cl concentration compared with the NaCl treatment. With the exception of a small reduction in *S. tomentosus*, this response was not present in inoculated plants (Figure 3.4b). In our earlier study (Franklin and Zwiazek 2004), chloride uptake and transport were independent of external concentration suggesting that rates are limited by the number of ion channels or carriers. The reduction in Cl shoot concentrations by B suggests that B may have interfered with Cl uptake or/and transport and that this factor was largely absent from the inoculated plants. However, I cannot speculate on the mechanisms which could have led to this response.

Root inoculation with mycorrhizal fungi had little effect on the concentrations and distribution of K in jack pine seedlings. However, treatments containing NaCl triggered an increase in shoot and decrease in root K levels (Figure 3.5a,b). The efflux of K from roots is likely an adaptive response to high external ion levels (Cumming and Taylor 1990) and was earlier reported for jack pine (Franklin and Zwiazek 2004), bean (*Phaseolus vulgaris*) (Carbonell-Barrachina et al. 1997) and radiata pine (Sands and Clarke 1977) treated with NaCl.

In summary, inoculation with mycorrhizal fungi increased dry weights, shoot:root dry weight ratios, and needle chlorophyll concentrations in jack pine seedlings, but had no effect on transpiration rates. The applied B treatment had little effect on growth and needle necrosis of plants, but induced small reductions in chlorophyll concentrations. When applied with NaCl, B aggravated needle necrosis and reduced Cl concentrations in shoots of non-inoculated plants. Plants treated with 2 mM H_3BO_3 + 60 mM NaCl had similar concentrations of Na and B to those that were treated separately with 60 mM NaCl and 2 mM H_3BO_3. Mycorrhization had relatively little effect on B concentrations in jack pine seedlings. However, mycorrhizal plants had lower shoot Na concentrations compared with non-inoculated seedlings when treated with NaCl and NaCl + B. Similarly, shoot Cl concentrations were reduced in mycorrhizal plants treated with NaCl, but NaCl + B treatment resulted in higher shoot Cl concentrations of inoculated jack pine seedlings compared with the non-inoculated plants.

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Table 3.1. ANOVA p-values for the main factors, their interactions. The main factors are: inocula (non-inoculated or inoculated), NaCl (0 and 60 mM NaCl treatments), boron (0 and 2 mM H_3BO_3). The measured parameters include total dry weights (DW), shoot to root ratios (S:R ratio), leaf chlorophyll concentrations (Chl), transpiration (E), necrosis (N) and root and shoot Na, Cl, B and K concentrations

	Total DW	S:R ratio	Chl	E	N	Root Na	Shoot Na	Root Cl	Shoot Cl	Root B	Shoot B	Root K	Shoot K
Inocula	<0.001	<0.001	<0.001	0.421	0.076	0.002	<0.001	0.104	0.425	0.001	<0.001	0.011	0.523
Boron	0.476	0.361	<0.001	0.032	<0.001	<0.001	<0.001	<0.001	<0.001	0.927	0.198	<0.001	<0.001
Inocula*boron	0.963	0.958	0.043	0.591	0.798	<0.001	<0.001	0.012	0.369	0.998	0.008	0.003	0.446
NaCl	<0.001	0.840	0.001	<0.001	<0.001	0.148	0.188	0.092	<0.001	<0.001	<0.001	0.620	0.092
Inocula*NaCl	0.024	0.454	0.494	0.986	0.039	0.087	0.099	0.375	<0.001	0.543	0.378	0.461	0.375
Boron*NaCl	0.145	0.065	0.507	0.015	0.618	0.245	0.231	0.474	<0.001	0.082	0.033	0.183	0.771
Inocula*Boron* NaCl	0.288	0.858	0.237	0.984	0.657	0.126	0.129	0.069	<0.001	0.717	0.864	0.291	0.054
Boron*NaCl †													
Non-Inoculated	0.071	0.517	0.032	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Hebeloma sp.	0.001	0.953	0.011	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
S.tomentosus	0.075	0.554	0.028	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
W. mikolae	0.998	0.352	<0.001	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Inocula ϕ													
Boron * NaCl	0.006	0.052	0.001	0.490	0.012	<0.001	<0.001	0.006	<0.001	0.085	<0.001	0.084	0.035
Boron * No NaCl	0.002	0.051	<0.001	0.513	0.980	0.734	0.998	0.878	0.982	0.146	0.458	0.204	0.715
No Boron * NaCl	0.012	0.113	0.002	0.894	0.086	<0.001	<0.001	0.021	<0.001	0.151	0.032	0.572	0.272
No Boron * No NaCl	<.001	0.078	0.003	0.892	1.000	0.376	0.998	0.218	0.999	0.272	0.475	0.001	0.637

† For each row above, a p-value less than 0.05 indicates a significant effect of boron and NaCl treatments for the inoculation treatment indicated

ϕ For each row above, a p-value less than 0.05 indicates a significant difference among inoculation treatments for the boron and NaCl treatment indicated

Table 3.2. Percentages of root lengths colonized with mycorrhizal fungi in non-inoculated jack pine seedlings and in seedlings inoculated with *Hebeloma* sp., *Wilcoxina mikolae* var. *mikolae* and *Suillus tomentosus* and subjected to 60 mM NaCl, 2 mM H_3BO_3 and 2mM H_3BO_3 + 60 mM NaCl treatments for four weeks. The values are means ± SE of 6 seedlings per treatment (n=6)

	Non-Inoculated	*Hebeloma sp.*	*Wilcoxina mikolae*	*Suillus tomentosus*
Non-Treated	26.6 ± 4.0	73.4 ± 4.6	75.7 ± 4.6	54.6 ± 2.9
NaCl	14.5 ± 2.8	66.1 ± 4.5	43.6 ± 4.5	52.4 ± 3.8
B	19.3 ± 4.6	55.8 ± 6.3	64.7 ± 4.1	50.6 ± 3.8
B + NaCl	15.5 ± 1.8	63.6 ± 3.8	46.4 ± 5.6	56.8 ± 8.4

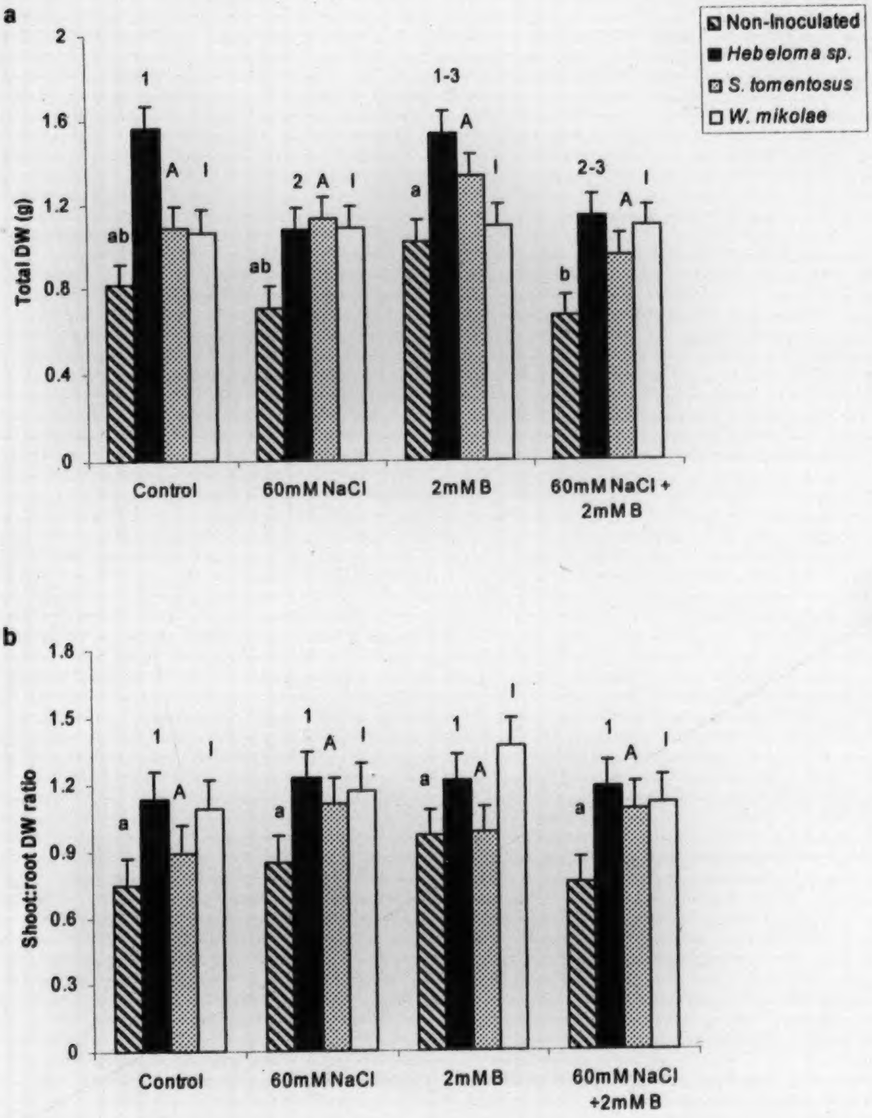

Figure 3.1. Total dry weights (a) and shoot:root ratios (b) in jack pine seedlings. The seedlings were either non-inoculated or inoculated with *Suillus tomentosus, Hebeloma* sp. or *Wilcoxina mikolae* var. *mikolae*. Each inoculation group was subjected to 2 mM H_3BO_3 and 60 mM NaCl separately and together (B + NaCl) for four weeks. Means (n = 8) + SE are shown. Different letters or numbers above the bars of the same inoculation treatment indicate significant differences ($\alpha = 0.05$) for different B and NaCl interaction combinations.

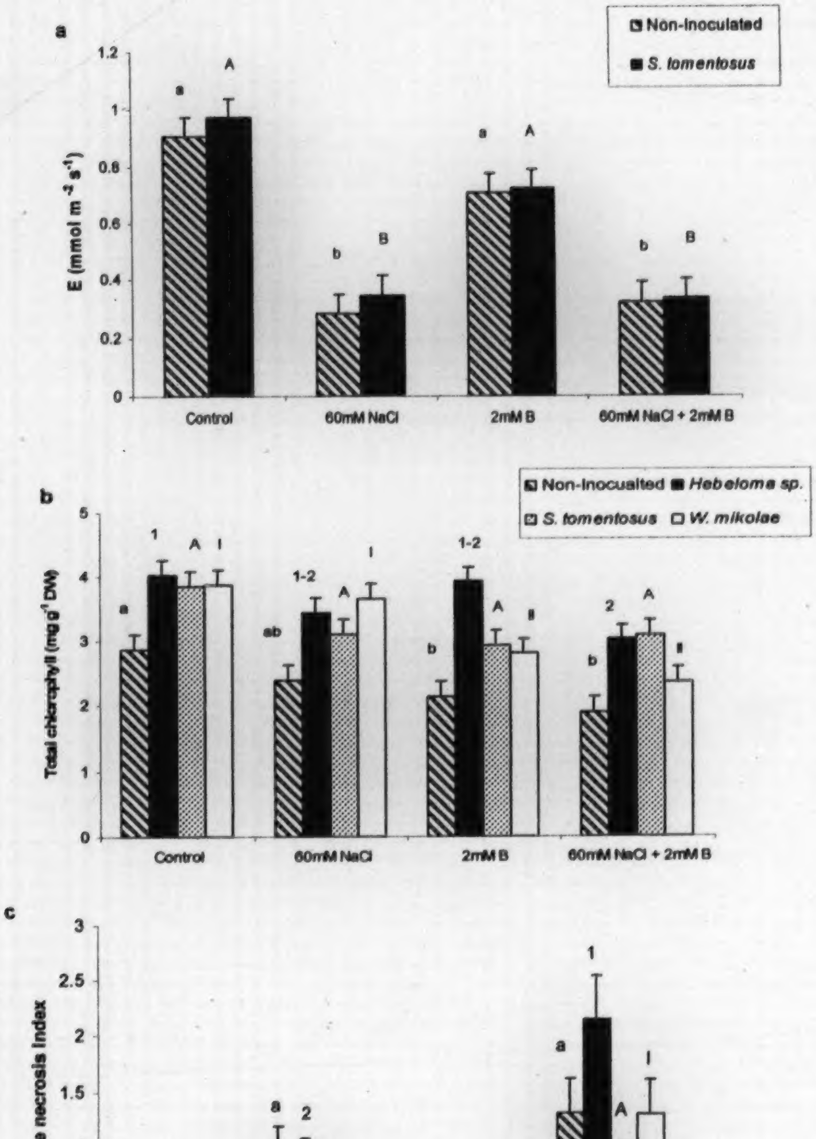

Figure 3.2. Transpiration rates (E) (a), needle chlorophyll concentrations (b) and necrosis index (c) in non-inoculated jack pine seedlings and in seedlings inoculated with *Suillus tomentosus Hebeloma* sp. or *Wilcoxina mikolae* var. *mikolae*. Each inoculation group was subjected to 2 mM H_3BO_3 and 60 mM NaCl separately and together (B + NaCl) for four weeks. Means (n = 8 for E and necrosis index, and n=6 for chlorophyll) + SE are shown. Different letters or numbers above the bars of the same inoculation treatment indicate significant differences ($\alpha = 0.05$) for different B and NaCl interaction combinations.

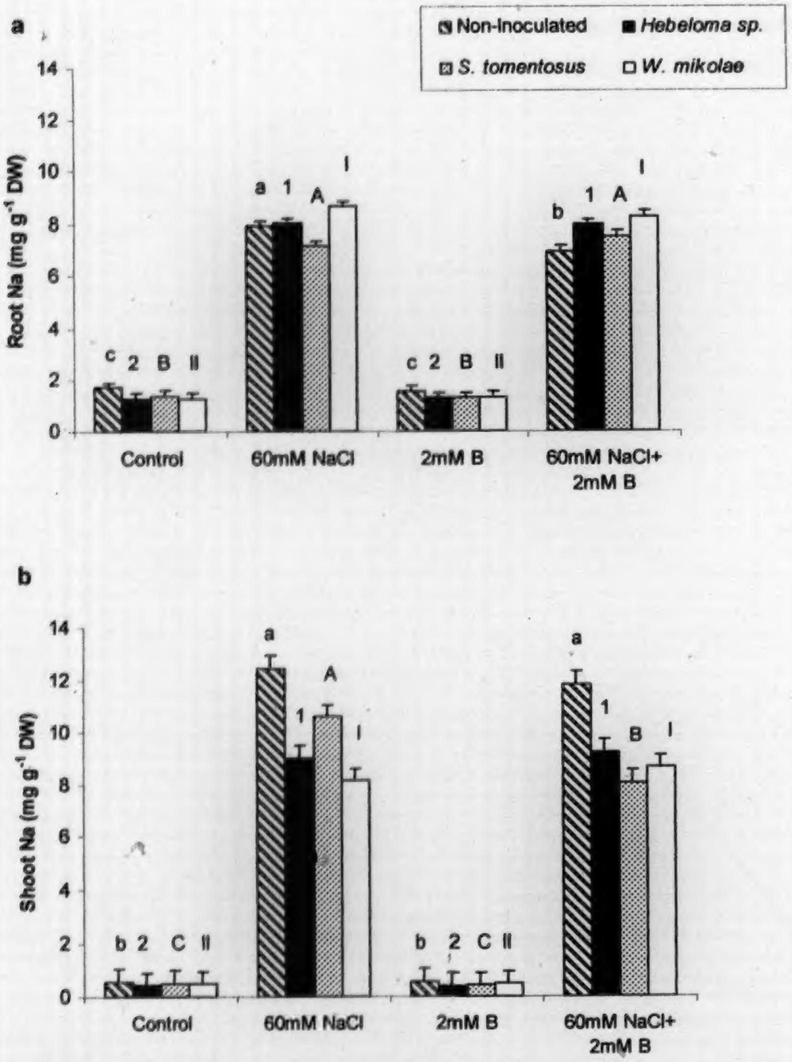

Figure 3.3. Concentrations of Na in roots (a) and shoots (b) of non-inoculated jack pine seedlings and in seedlings inoculated with *Suillus tomentosus Hebeloma* sp. or *Wilcoxina mikolae* var. *mikolae*. Each inoculation group was subjected to 2 mM H_3BO_3 and 60 mM NaCl separately and together (B + NaCl) for four weeks. Means (n = 6) + SE are shown. Different letters or numbers above the bars of the same inoculation treatment indicate significant differences ($\alpha = 0.05$) for different B and NaCl interaction combinations.

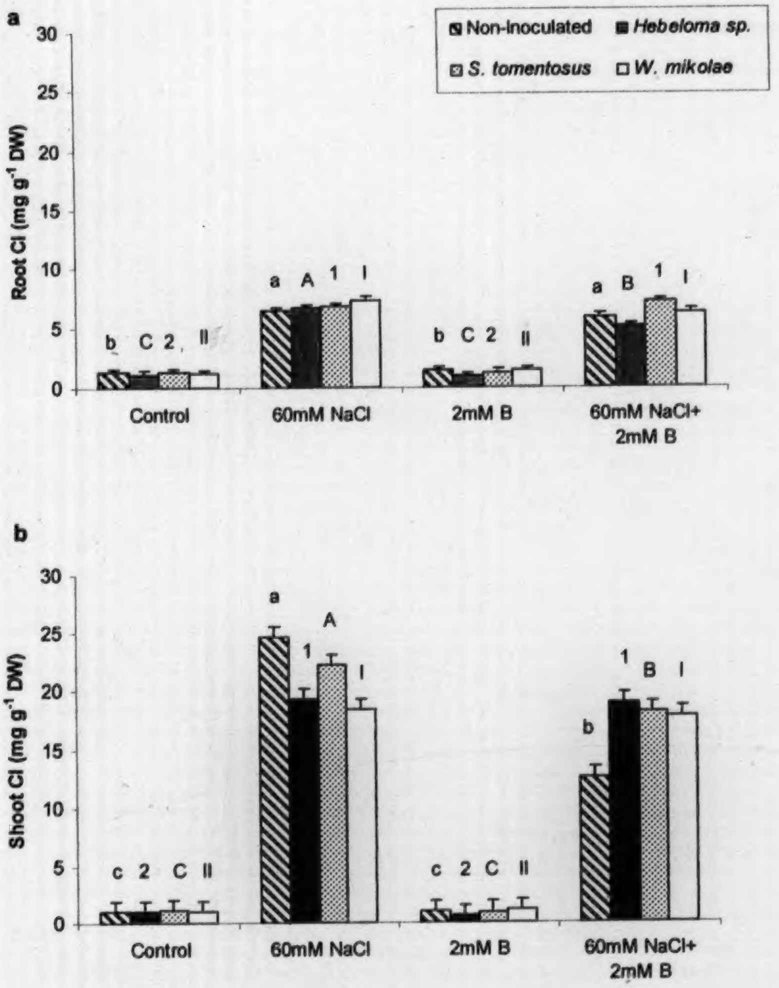

Figure 3.4. Concentrations of Cl in roots (a) and shoots (b) of non-inoculated jack pine seedlings and in seedlings inoculated with *Suillus tomentosus Hebeloma* sp. or *Wilcoxina mikolae* var. *mikolae*. Each inoculation group was subjected to 2 mM H_3BO_3 and 60 mM NaCl separately and together (B + NaCl) for four weeks. Means (n = 6) + SE are shown. Different letters or numbers above the bars of the same inoculation treatment indicate significant differences ($\alpha = 0.05$) for different B and NaCl interaction combinations.

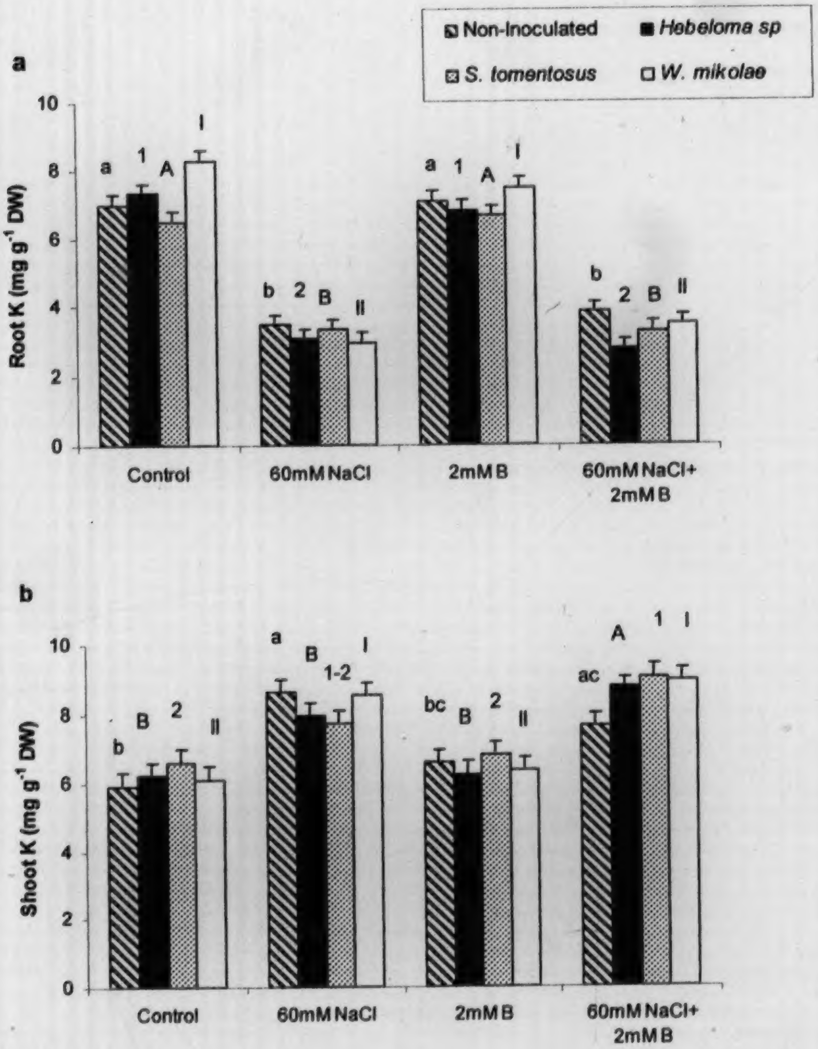

Figure 3.5. Concentrations of K in roots (a) and shoots (b) of non-inoculated jack pine seedlings and in seedlings inoculated with *Suillus tomentosus Hebeloma* sp. or *Wilcoxina mikolae* var. *mikolae*. Each inoculation group was subjected to 2 mM H_3BO_3 and 60 mM NaCl separately and together (B + NaCl) for four weeks. Means (n = 6) + SE are shown. Different letters or numbers above the bars of the same inoculation treatment indicate significant differences ($\alpha = 0.05$) for different B and NaCl interaction combinations.

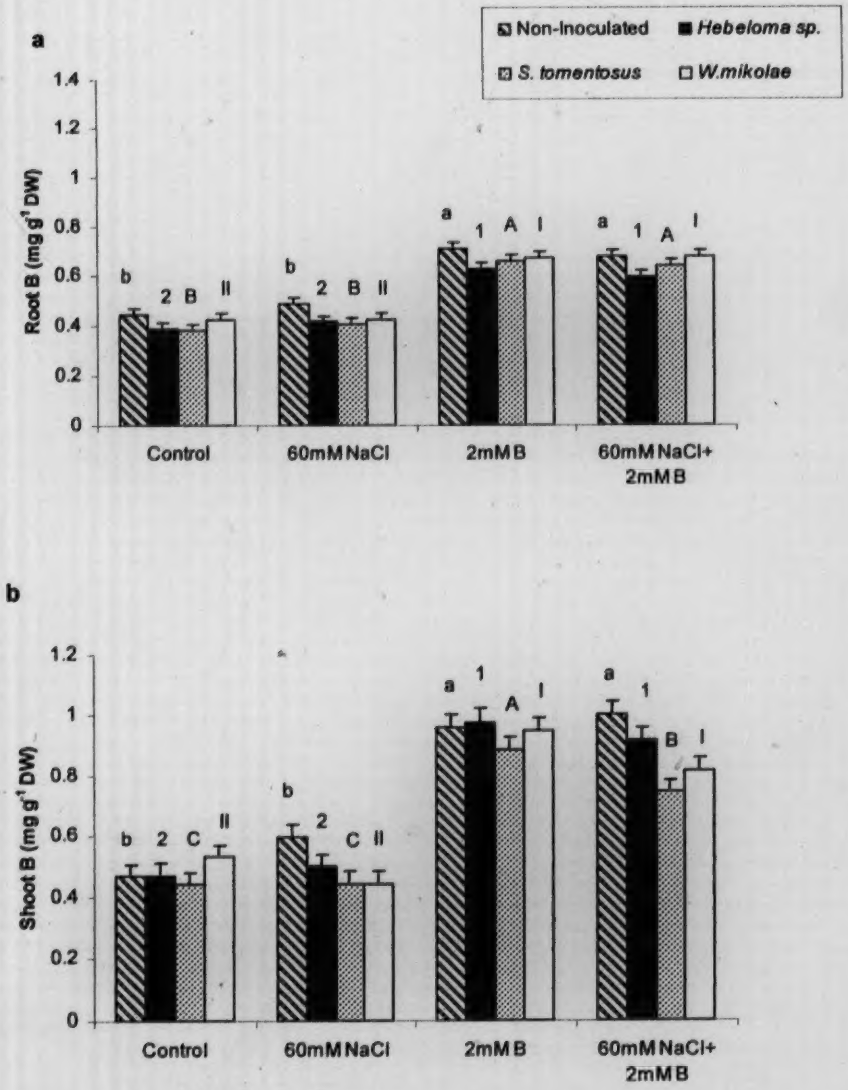

Figure 3.6. Concentrations of B in roots (a) and shoots (b) of non-inoculated jack pine seedlings and in seedlings inoculated with *Suillus tomentosus Hebeloma* sp. or *Wilcoxina mikolae* var. *mikolae*. Each inoculation group was subjected to 2 mM H_3BO_3 and 60 mM NaCl separately and together (B + NaCl) for four weeks. Means (n = 6) + SE are shown. Different letters or numbers above the bars of the same inoculation treatment indicate significant differences ($\alpha = 0.05$) for different B and NaCl interaction combinations.

CHAPTER IV*: Responses of ectomycorrhizal American elm (*Ulmus americana*) seedlings to salinity and soil compaction

4.1 Introduction

Soil compaction and pollution due to heavy pedestrian and vehicle traffic make urban environments among the most challenging places for vegetation growth. The problem is aggravated by the presence of salts, often due to winter de-icing of city roads (Lait et al. 2001, Kayama et al. 2003, Cunningham et al. 2008) which can result in soil electrical conductivities as high as 9 dS m^{-1} in some urban locations (Calvo and Zwiazek, unpublished data). Since urban trees have high esthetic, recreational and economic value, their protection is a major issue.

Salt affects plants by inducing water stress, nutrient imbalance and ion toxicity (Munns 1993, Czerniawska-Kusza et al. 2004, Franklin and Zwiazek 2004). Salt resistance mechanisms in plants may be metabolically-dependent (Redfield et al. 2003) since Na exclusion mechanisms include metabolically-controlled membrane transport processes (Drew and Läuchli 1985, Barrett-Lennard et al. 2003). Hypoxia may increase the entry of Na into roots and allow more NaCl to move into the xylem apoplastically (Barrett-Lennard et al. 2003). Root hypoxia also inhibits the function of root aquaporins and reduces root water uptake (Zhang and Tyerman 1991). Since soil compaction decreases pore size and reduces soil oxygen content, it effectively creates hypoxic conditions for roots (Mariani et al. 2006, Haeussler and Kabzems 2005). Therefore, soil compaction may potentially

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The ability of American elm (*Ulmus americana* L.) to tolerate urban environments and its high ornamental value make it one of the most valuable urban trees in North America (Bey 1990). American elm has a root system that consists of numerous short lateral roots that typically contains vesicular -arbuscular (VA) Paris-type mycorrhizal associations (Brundett et al. 1990). However, elm trees can also form ectomycorrhizal (ECM) associations with some species of fungi including *Hebeloma crustuliniforme* [Bull.] Quel. (Muhsin and Zwiazek 2002a). It is well established that ECM fungi improve water (Muhsin and Zwiazek 2002a,b; Landhäusser et al. 2002) and nutrient (Jones et al. 1990) uptake and decrease plant uptake of salts (Muhsin and Zwiazek 2002a) and metals (Jones and Hutchinson 1988). Since the diameter of fungal hyphae is smaller than the diameter of the finest roots, the hyphae could more easily penetrate small pores in compacted soils and access the water and nutrient resources (Schack-Kirchner et al. 2000, Allen 2007). Therefore, ECM associations could benefit trees growing in urban soils that are affected by salts and compaction.

Hebeloma crustuliniforme and *Laccaria bicolor* [R. Maire] Orton are pioneer ECM species that are associated with a wide range of host trees. Our earlier studies showed that these ECM fungi reduced salt uptake by shoots (Muhsin and Zwiazek 2002b, Nguyen et al. 2006), however, it is not clear if these associations are also effective in compacted soils.

In the present study, I examined the effects of soil compaction on salt tolerance of American elm (*Ulmus americana*) seedlings that were inoculated with the ECM fungi *Hebeloma crustuliniforme* and *Laccaria bicolor*. Since the ECM fungi may be able to penetrate small pores in the compacted soil, they could access oxygen and other soil resources that may not be available to non-mycorrhizal plants. Therefore, I hypothesized that ECM plants would also be more resistant to salt stress than non-mycorrhizal plants when subjected to soil compaction.

4.2 Materials and Methods

4.2.1 Plant material and growth conditions

American elm (*Ulmus americana*) seeds were collected at the University of Alberta campus (Edmonton, Alberta, Canada) during the spring of 2005. The seeds were surface-sterilized with 3% H_2O_2 for 30 s and germinated in Jiffy-7 pellets (Jiffy Products Ltd, Shippegan, NB Canada) in June, 2005. The pellets were placed in a growth room set to 20/16°C day/night temperature, 16-h photoperiod, 65±10% relative humidity and 350 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at the seedling level. Six weeks after germination, the seedlings were transferred to 700 cm^3 styroblock containers (Beaver Plastics Ltd, Acheson, AB, Canada) filled with a compacted or non compacted (see below) mixture of peat moss, sand and white fine clay (3:1:0.75 v/v/v). Before adding to the containers, the soil mixture had been sterilized by autoclaving 2 x 20 min at 121°C over two days. Mineral nutrients (20:20:20 N:P:K commercial fertilizer) were applied to the seedlings every two weeks at a 1g L^{-1} concentration. The soil was flushed weekly with de-ionized water to prevent ion accumulation. Starting at the end of August 2005, the containers with seedlings were moved outside for winter. In March 2006, the seedlings were moved to the greenhouse that was set to the same conditions as the growth chamber above except that the natural light was supplemented by 400-W high pressure sodium lamps (Lumalux; GTE Sylvania, Drummondville, PQ, Canada).

4.2.2 Fungal inoculation

The fungal cultures consisted of *Laccaria bicolor*, UAMH 8232*)*, *Hebeloma crustiliniforme* (UAMH 5247), and a mixture (1:1 v/v) of *L. bicolor* and *H. crustuliniforme*. A non-inoculated group of seedlings to which water was added served as a control. The inoculum was prepared by growing fungal pure culture in a modified Melin-Norkans (MMN) liquid medium (Mason 1980) for four weeks in 4 cm (diameter) x 60 cm custom-made glass cylindrical flasks. Each flask contained about 1.5 l of liquid medium. The cultures were constantly aerated with a pump connected to the side arm of the glass flask through the PVC tubing. The seedlings were inoculated with fungal cultures when they were one-month-old and for the second time one day after being moved to the greenhouse in March 2006. For the first inoculation, 25 ml of each fungal culture was diluted to 500 ml with deionized water. For the second inoculation, two 5-cm-deep holes were made in the soil on both sides of each seedling and 10 ml of inoculum (prepared as above except that diluted only 1:1 (v/v) with water) was applied to the each hole. Roots were checked for fungal colonization at the end of NaCl and soil compaction treatments. Six root tips were removed from each of the 6 seedlings per treatment combination and preserved in ethanol-glycerol-water solution (4:3:3 v/v/v). Distal, 1 to 2-cm long, root segments were cleared with 10% KOH, stained with 5% black ink-vinegar solution (Vierheilig et al. 1998) and examined with a light microscope.

4.2.3 Soil compaction and salt treatments

For the compaction treatment, one-third of the containers were filled with the soil and the soil was compacted by dropping a 3-kg metal weight three times from the 60-cm height. The treatment was repeated two more times for each pot after adding more soil to the two-thirds and full pot capacity, resulting in a 50% increase in soil bulk density from 0.4 g cm^{-3} in non-compacted to 0.6 g cm^{-3} in compacted soil. The compaction treatment was chosen to represent moderately to moderately-heavy compacted soil. Thirty-two seedlings of the each inoculation treatment and 32 non-inoculated seedlings were transferred to 700 cm^3 pots with either compacted or non-compacted soil, with a total of 128 pots.

Salt treatments were applied for three weeks starting in June, three months after the second fungal inoculation. Sixteen seedlings were selected from each fungal and compaction treatment combination. Half of the seedlings from each group were subjected to salt treatment and the other half served as no salt control. For the salt treatment, pots with the seedlings were immersed every four days for 24 h in 60mM NaCl or water (no salt-control). The soil was flushed once a week to prevent salt build-up. During the NaCl treatment, seedlings were fertilized every two weeks by adding 20:20:20 (N:P:K) to NaCl treatment solution (water for control seedlings).

4.2.4 Dry weights, gas exchange, leaf chlorophyll content and bud flushing

Shoot and root dry weights were determined for eight seedlings per treatment combination (n=8). Roots and stems were dried in an oven at 65°C for 48 hours. To determine shoot dry weights, leaves were freeze-dried for 48 hours and their weights added to the weights of the stems.

Net photosynthesis (NP) and transpiration (E) rates were measured in the uppermost fully developed leaves of eight seedlings per treatment combination (n=8). An infrared gas analyzer (LCA-4, Analytical Development Company Ltd., Hertfordshire, UK) with an auxiliary LED bulb (1200 µmol m^{-2} s^{-1} PPFD) were used for the measurements as previously described (Voicu et al. 2008). Leaf areas were calculated with the LI-3000 leaf area meter (LI-COR Biosciences, Lincoln, NE, USA).

For chlorophyll a and b determinations, freeze-dried leaves from each seedling were ground and 20 mg samples taken for chlorophyll extraction (n=6). Chlorophyll was extracted with 8 ml dimethyl sulfoxide (DMSO) for 26 hours in an oven at 65°C and the extracts analyzed spectrophotometrically (Ultrospec III, Pharmacia LKB, Uppsala, Sweden). Total chlorophyll was calculated using the Arnon's equation (Šesták et al. 1971).

The seedlings were checked weekly for the signs of bud flushing for six weeks after being transferred to the greenhouse. A seedling with a minimum of one open bud containing visible new green leaves was considered flushed.

4.2.5 Root hydraulic conductance (K_r) *and leaf hydraulic conductance* (K_L)

A high pressure flow meter (HPFM, Dynamax Inc., Houston, TX, USA) was used for the measurements of root hydraulic conductance (K_r) (Muhsin and Zwiazek, 2002b). The shoots of seven seedlings (n=7) per treatment combination were excised 1.5 cm above the root collar and the roots attached through the cut stem to the HPFM and pressurized up to 0.5MPa for the measurements of K_r.

To measure leaf hydraulic conductance, shoots were attached to the HPFM and flushed with water at constant pressure until the flow became relatively stable (Voicu et al. 2008). A computer recorded the flow and saved it as a mean every 60s. The resistance of the whole shoot (R_S, MPa s kg^{-1}) was computed as the mean value of the last six stable readings. After the resistance of the whole-shoot was determined, leaves were detached and a new stable flow was recorded. The resistance of the leafless shoot (R_{SL}, MPa s kg^{-1}) was determined similarly, as the mean value of the last six stable readings. Leaf area (A, m^2) was measured as above. Leaf hydraulic conductance was calculated as: $K_L=(1/(R_S-R_{SL}))/A$ (kg s^{-1} MPa^{-1} m^{-2}). Leaf hydraulic conductance was measured in plants of the non-compacted soil treatments with seven replicates per treatment (n=7).

4.2.6 Tissue Na concentrations

After three weeks of 60 mM NaCl treatment, six seedlings (n=6) per treatment combination were selected for Na analyses. Roots were excised and the leaves were separated from stems and dried for 48 hours. Liquid nitrogen was added to the dried root and leaf samples and the samples were ground in a mortar to fine powder.

Ground tissue samples (0.5-1 g) were used for the dry ashing extraction (Richards 1993). The Na concentrations in tissue extracts were determined with a Model AA880 Atomic Absorption Spectrophotometer (Varian Inc., Mississuaga, ON, Canada).

4.2.7 Experimental design and statistical analysis

The experiment was a 4x2x2 complete randomized factorial design with three fungal treatments plus a control non-inoculated treatment, two soil compaction levels (compacted vs. non-compacted) and two salt levels (60mM NaCl and 0mM NaCl). The analysis of variance was performed using the Mixed Procedure of SAS (Version 9.1, SAS Institute Inc.; Cary, NC, USA). To meet the assumptions of of normality of distribution and homogeneity of variance of ANOVA, the data for root hydraulic conductance and Na tissue concentrations were transformed with a

$\log_{10}$ function. The values for the transformed means and their standard errors were back-transformed to present in the figures. The SLICE option from SAS (Littell et al. 2006) was used to examine the effects of the inoculation treatment (Non-Inoculated, *H. crustuliniforme*, *L .bicolor* and *H. crustuliniforme + L. bicolor*) with the Compaction * NaCl interaction and the effects of the interaction of Compaction*NaCl treatments at each inoculation level (Table 4.1). Tukey's adjustment was used for paired-comparisons among treatment means at α=0.05. ANOVA p-values for the main factors, their interactions, and the effects of Compaction*NaCl treatment combinations within and across each inoculation group are shown in Table 4.1.

The statistical models for the experiment were as follows:

$Y_{ijkl} = \mu + M_i + N_j + C_k + M_iN_j + M_iC_k + N_jC_k + M_iN_jC_k + e_{ijkl}$, where

Y_{ijkl} = value of individual observation (i = mycorrhiza, j = NaCl, k = compaction l=observation)

μ = overall mean of observations

M_i = effect of i^{th} treatment (i = mycorrhizal treatment)

N_j = effect of j^{th} treatment (j = NaCl treatment)

C_k = effect of k^{th} treatment (k= compaction treatment)

M_iN_j = interaction effect of i^{th} and j^{th} treatments

M_iC_k = interaction effect of i^{th} and k^{th} treatments

N_jC_k= interaction effect of j^{th} and k^{th} treatments

$M_iN_jC_k$= interaction effect of i^{th}, j^{th} and k^{th} treatments

e_{ijkl} = residual error

For the K_L measurements:

$Y_{ijk} = \mu + M_i + N_j + M_iN_j + e_{ijl}$, where

Y_{ijk} = value of individual observation (i = mycorrhiza, j = NaCl, k=observation)

μ = overall mean of observations

M_i = effect of i^{th} treatment (i = mycorrhizal treatment)

N_j = effect of j^{th} treatment (j = NaCl treatment)

M_iN_j = interaction effect of i^{th} and j^{th} treatments

e_{ijk} = residual error

4.3 Results

4.3.1 Root colonization, plant dry weights and timing of bud flushing

Although the surfaces of the inoculated roots were largely covered with a mass of loosely-organaized hyphae, the roots did not develop a well defined mantle. The leaves of inoculated seedlings became chlorotic during the first month following the first inoculation, but recovered over time.

Compaction and NaCl treatments significantly reduced plant dry weights in non-inoculated seedlings ($p = 0.003$ and $p = 0.018$, respectively). There was a further reduction in dry weights of soil compacted plants when treated with NaCl in seedlings inoculated with *L. bicolor* ($p = 0.005$), but not in plants inoculated with *H. crustuliniforme* ($p = 0.292$) or *H. crustuliniforme* + *L. bicolor* ($p = 0.180$). In untreated seedlings, inoculation with EM fungi affected dry weights differently depending on the inoculation treatment. *Hebeloma crustuliniforme* reduced total dry weights ($p = 0.049$), but there was no significant reduction of dry weights in seedlings inoculated with *L. bicolor* and a mixture of both fungi (Figure 4.1a). Compaction treatment had no effect on the dry weights of seedlings inoculated with the EM fungi (Figure 4.1a). Similarly, NaCl treatment had no significant effect on the dry weights in seedlings inoculated with *H. crustuliniforme* and a mixture of *H. crustuliniforme* + *L. bicolor* (Figure 4.1a).

There was about 20-30% reduction in shoot to root dry weight ratios in non-inoculated plants growing in compacted compared with non-compacted soil (Figure 4.1b). Similar reductions were also measured for plants inoculated with *L. bicolor*, but not for *H. crustuliniforme* and the mixture of both fungi (Figure 4.1b).

Bud flushing started one week after the seedlings were moved to the greenhouse. Compaction and inoculation treatments delayed bud flushing (Figure 4.2a,b). At week one, bud flushing occurred in 15-37% of non-compacted inoculated plants (Figure 4.2a) and at 30% of the non-inoculated seedlings in compacted soil (Figure 4.2b). In contrast, 64% of non-inoculated seedlings from the non-compacted treatment had opened buds at that time (Figure 4.2b).

4.3.2 Chlorophyll contents and gas exchange

Net photosynthesis (NP) (Figure 4.3a) and transpiration rates (E) (Figure 4.3b) followed a similar trend in all experimental groups. In untreated seedlings, mycorrhizal inoculation had little effect on the measured gas exchange parameters (Figure 4.3a,b). In non-inoculated plants, soil compaction and salt treatments reduced NP ($p = 0.002$) and E ($p < 0.001$) to less than 50% of the values measured in untreated control (Figure 4.3a,b). There were only small, statistically insignificant effects of NaCl and compaction treatments on both gas exchange parameters in inoculated plants when the treatments were applied separately (Figure 4.3a,b). However, when plants growing in compacted soil were subjected to NaCl treatment, there was a further decline in E and NP in inoculated plants compared with non-inoculated seedlings (Figure 4.3a,b).

There were no visual chlorotic or necrotic lesions in leaves of seedlings during the length of the experiment. However, mycorrhizal inoculation reduced leaf chlorophyll concentration in non-treated plants (Figure 4.3c). Soil compaction and NaCl treatments had little effect on leaf chlorophyll contents (Figure 4.3c).

4.3.3 Root and leaf hydraulic conductance

In untreated (non-compacted, 0 mM NaCl) plants, fungal inoculation reduced root hydraulic conductance (K_r) by about two-fold compared with non-inoculated plants (Figure 4.4). Similar reductions in non-inoculated plants ($p < 0.001$) were also due to NaCl and compaction treatments (Figure 4.4). In all three groups of inoculated seedlings (H. *crustuliniforme, L. bicolor* and *H. crustuliniforme* + *L. bicolor*), soil compaction had no effect on K_r (Figure 4.4). The response of K_r to NaCl varied between the different inoculation treatments. In plants inoculated with *H. crustuliniforme*, K_r was significantly reduced by the NaCl treatment ($p = 0.001$) (Figure 4.4). However, in plants inoculated with *L. bicolor* and *H. crustuliniforme* + *L. bicolor*, there were only small, statistically insignificant, reductions in K_r (Figure 4.4). Soil compaction + NaCl treatment reduced K_r in plants inoculated with *H. crustuliniforme* and *L. bicolor* (Figure 4.4).

Effect of NaCl on leaf hydraulic conductance (K_L) was measured in non-inoculated plants and plants inoculated with *H. crustuliniforme* and subjected to 0 and 60 mM NaCl treatment (non-compacted soil). There were small reductions in

K_L by salt that was measured in non-inoculated plants (2. 12 ± 0.19 kg s^{-1} $MPa^{-1}m^{-2}$ x 10^{-4} for no salt and 1.63 ± 0.19 kg s^{-1} $MPa^{-1}m^{-2}$ x 10^{-4} for salt-treated plants, mean ± SE, n = 7) (p = 0.072) and in plants that were inoculated with *H. crustuliniforme* (2. 19 ± 0.19 kg s^{-1} $MPa^{-1}m^{-2}$ x 10^{-4} for no salt and 1.99 ± 0.19 kg s^{-1} $MPa^{-1}m^{-2}$ x 10^{-4} for salt-treated plants, mean ± SE, n = 7) (p = 0.508).

4.3.4 Na tissue concentrations

Mean Na concentrations in the leaves of non-inoculated seedlings were between about fourfold and 14- fold higher compared with NaCl-treated inoculated plants ($p < 0.001$) (Figure 4.5a). Soil compaction reduced Na concentrations by several-fold in leaves of non-inoculated plants ($p < 0.001$) (Figure 4.5a). The reduction in Na concentrations by soil compaction did not occur in the inoculated plants (Figure 4.5a). Tissue Na concentrations in roots were similar in all inoculated and non-inoculated plants subjected to NaCl and NaCl + soil compaction treatments (Figure 4.5b).

4.4 Discussion

After the first inoculation (seedlings one-month old), the inoculated plants showed signs of chlorosis and reduced growth (data not shown). The reductions in weights and leaf chlorophyll concentrations were still present in inoculated non-treated plants at the time of measurements (Figure 4.1a, 4.3c). In different studies, ECM fungi increased (Muhsin and Zwiazek 2002a; Nguyen et al. 2006), decreased (Corrêa et al. 2006; Nguyen et al. 2006) or had no effect (Thomson et al. 1994; Landhäusser et al. 2002; Corrêa et al. 2006) on plant growth. The effect of ECM fungus may depend on the age, stage of development, and nutritional status of plants (Corrêa et al. 2006).

In our study, plant responses to the applied treatments followed similar patterns in the three studied fungal inocula (*H. crustuliniforme*, *L. bicolor* and *H. crustuliniforme* + *L. bicolor*). A decrease in dry weights of untreated ECM plants was accompanied by a decrease in root hydraulic conductance (K_r) but there was little effect on leaf hydraulic conductance (K_L) and net photosythesis (NP). In most studies, ECM resulted in an increase in K_r (Muhsin and Zwiazek 2002a,b; Landhäusser et al. 2002; Marjanović et al. 2005), however, no effects of ECM on K_r have also been reported (Nardini et al. 2000; Yi et al. 2008). Hydraulic conductance is affected by the size of the root system (Muhsin and Zwiazek 2002a). However, when I expressed K_r on the root volume basis, the results were similar (data not shown). The effects of the ECM associations on root hydraulic conductance are likely due to their effects on aquaporin expression (Marjanović et al. 2005). It has been pointed out that a reduction of K_r could be due to the parasitic effect of the ECM fungus (Yi et al. 2008). *Ulmus americana* is commonly associated with the vascular-arbuscular mycorrhizas (VAM). It is plausible that in my study, inoculation with ECM fungi increased the demand on energy resources of the elm seedlings. This could be partly responsible for the observed delay in bud flushing of inoculated plants and in the plants subjected to soil compaction (Figure 4.2). Also, the K_r values that were measured in non-inoculated elm seedlings were already likely affected by the presence of VAM (Aroca et al. 2007; Porcel et al. 2007). It is interesting that the ECM fungi did not affect net photosynthesis but decreased plant dry weights. A likely explanation is that the photosynthates were supplied to the fungus which may be beneficial to plants when they are exposed to stress. Alternatively, photosynthetic and growth reductions could have occurred only initially following seedling inoculation with the ECM fungi.

Salt inhibits growth and affects water relations and gas exchange in plants (Munns 1993; López-Berenguer et al. 2006). In the present study, ECM fungi reduced leaf Na uptake by elm seedlings when treated with NaCl in non-compacted soil (Figure 4.5a). It was previously reported that Na shoot uptake was reduced in the studied ECM conifer seedlings (Muhsin and Zwiazek 2002b; Bois et al. 2006; Nguyen et al. 2006), but not in *Betula papyrifera* and *Populus tremuloides* (Yi et al. 2008). The exact mechanisms of the reduction in salt uptake by shoots are not known, but they may include exclusion and/or sequestration of Na by the fungal hyphae (Muhsin and Zwiazek 2002b).

Compaction decreases large pores in the soil and reduces the availability of water and oxygen to plants (Haeussler and Kabzems 2005; Mariani et al. 2006). In our study, compaction reduced shoot and root dry weights, transpiration rates, net photosynthesis as well as root hydraulic conductance in non-inoculated, but not in inoculated plants. It was reported that the ECM fungal hyphae can occupy smaller soil pores than the finest plant roots (Allen 2007). On the other hand, the ectomycorrhizae of trees are highly oxygen demanding (Slankis 1974) and their growth can be mechanically restricted (Skinner and Bowen 1974). Therefore, further studies are needed to explain the effects of ECM that I have reported in the present study. Our results clearly demonstrate that ECM associations could be highly beneficial to plants growing in sites with compacted soil such as urban areas.

Both NaCl and compaction treatments reduced K_r by up to four-fold in non-inoculated seedlings (Figure 4.4a). However, compaction did not have statistically significant effect on K_r in ECM plants. This suggests that the salt and compaction affect the root water flow properties through different mechanisms. Although I did not measure the effect of compaction on K_L, there was a small reduction in K_L in NaCl-treated non-inoculated plants, but NaCl-treated inoculated plants. Leaf water transport has been long viewed as a largely apoplastic process of water movement. However, recent studies have demonstrated that aquaporin-mediated cell-to-cell water transport may also play a significant role in leaves (Cochard et al. 2007). Therefore, it is possible that the effects of NaCl on K_L may involve aquaporin-mediated water transport processes.

Salt exclusion capabilities of plants appear to be energy-dependent and are affected by hypoxic conditions (Drew and Lauchli 1985; Barrett-Lennard et al. 2003; Apostol and Zwiazek 2003). I anticipated that ECM would still be able to reduce Na leaf uptake by plants growing in compacted soil. In my study, root Na concentration in ECM and non-ECM seedlings were not affected by soil compaction, however, the leaf Na concentrations were reduced by several-fold in all soil compaction + NaCl treatments. The results suggest that soil compaction affected plants differently than root hypoxia that was applied in solution culture experiments where the ability of plants to sequester Na was reduced (Apostol and Zwiazek 2003; Redfield et al. 2003). It is possible that the reduction in plant water uptake (reduced transpiration rates and root hydraulic conductance) together with lower growth rates could have contributed to the reduced Na transport to the leaves (Shennan et al. 1987).

In conclusion, I have found that all three fungal inocula had similar effects on the responses of elm seedlings to soil compaction and salt treatments. When grown in non-compacted soil, ECM fungi reduced plant dry weights and root hydraulic conductance, but did not affect leaf hydraulic conductance and net photosynthesis. ECM seedlings treated for three weeks with 60 mM NaCl had several-fold lower Na concentrations in the leaves compared with non-inoculated plants. Soil compaction reduced Na leaf concentrations by non-ECM plants and decreased dry weights, gas exchange and root hydraulic conductance. However, in ECM plants, soil compaction had little effect on Na leaf concentrations and on the measured growth and physiological parameters.

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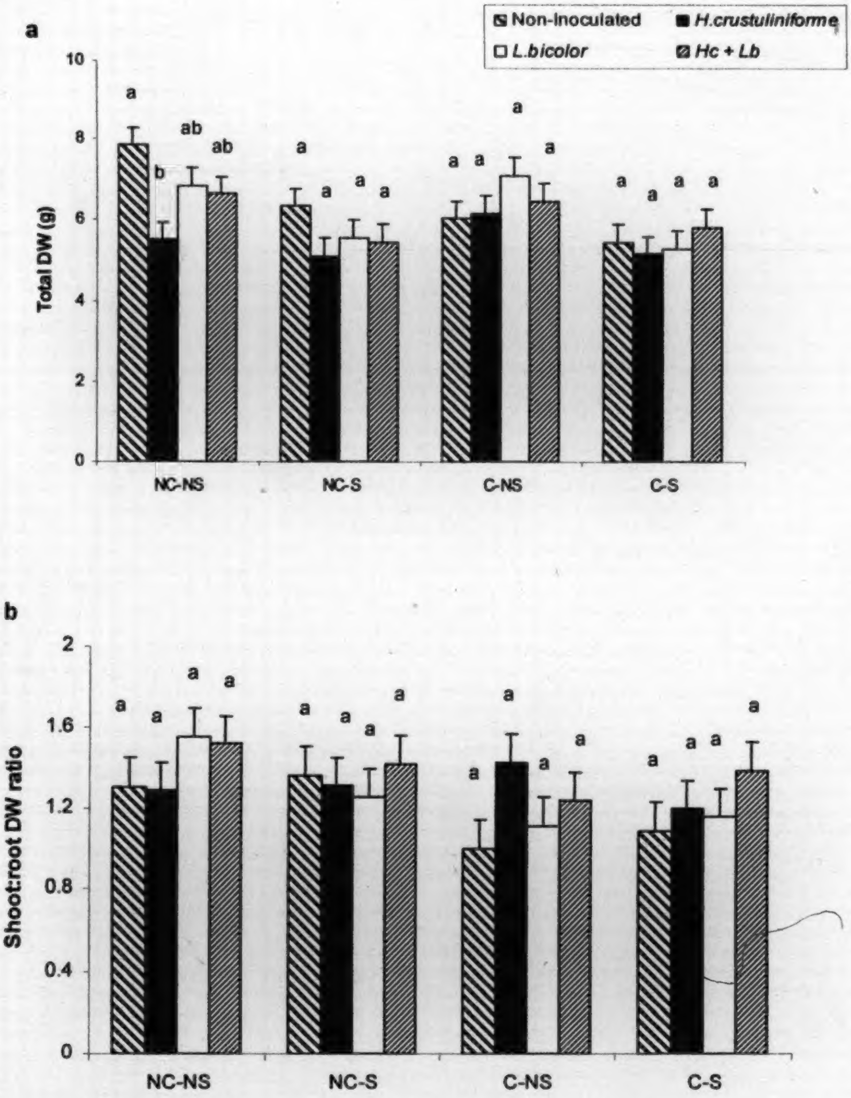

Figure 4.1. Total dry weights (a) and shoot:root ratios (b) in American elm seedlings. The seedlings were either non-inoculated or inoculated with *Hebeloma crustuliniforme* (Hc), *Laccaria bicolor* (Lb) or a mixture of *Hebeloma crustuliniforme* and *Laccaria bicolor* (Hc+Lb). Each group was subjected to soil compaction (C) or no compaction (NC) treatments and 0 mM NaCl (NS) or 60 mM NaCl (S). Means (n = 8) and standard errors are shown. Letters above the bars indicate significant differences ($\alpha = 0.05$) within each treatment group (compaction-salt treatment combination within each inoculum treatment)..

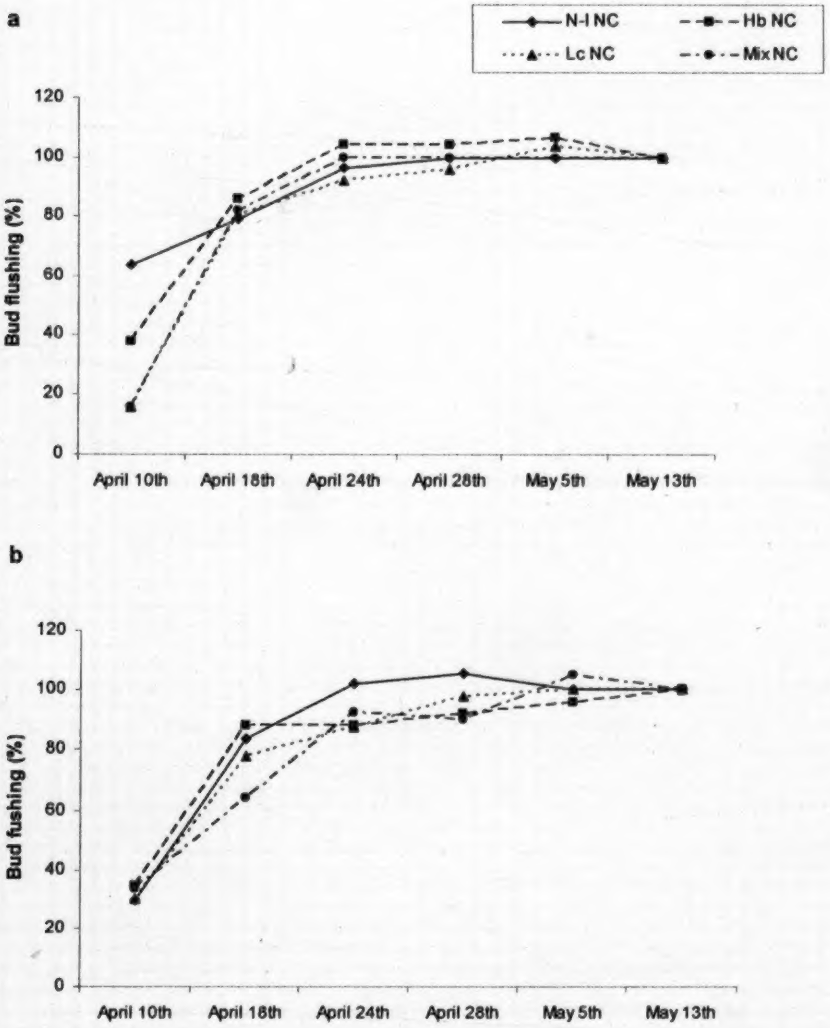

Figure 4.2. Timing of bud flushing in American elm seedlings growing in non-compacted (a) and compacted (b) soil after transferring the seedlings to the greenhouse in early spring. The seedlings were either non-inoculated (N-I) or inoculated with *Hebeloma crustuliniforme* (Hc), *Laccaria bicolor* (Lb) or a mixture of *Hebeloma crustuliniforme* and *Laccaria bicolor* (Hc+Lb).

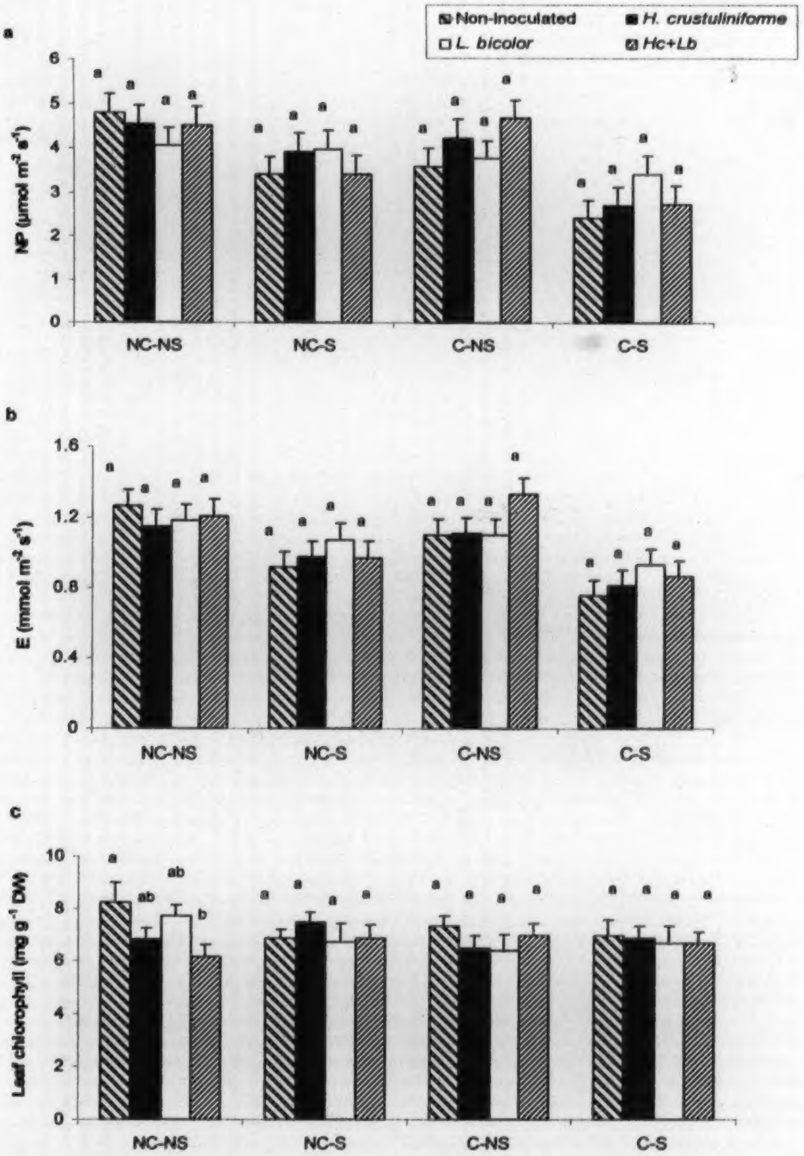

Figure 4.3. Net photosynthesis (NP) (a), transpiration rates (E) (b) and leaf chlorophyll concentrations (c) in American elm seedlings. The seedlings were either non-inoculated (N-I) or inoculated with *Hebeloma crustuliniforme* (Hc), *Laccaria bicolor* (Lb) or a mixture of *Hebeloma crustuliniforme* and *Laccaria bicolor* (Hc+Lb). Each group was subjected to soil compaction (C) or no compaction (NC) treatments and 0 mM NaCl (NS) or 60 mM NaCl (S). Means (n = 8) and standard errors are shown. Letters above the bars indicate significant differences (α = 0.05) within each treatment group (compaction-salt treatment combination within each inoculum treatment).

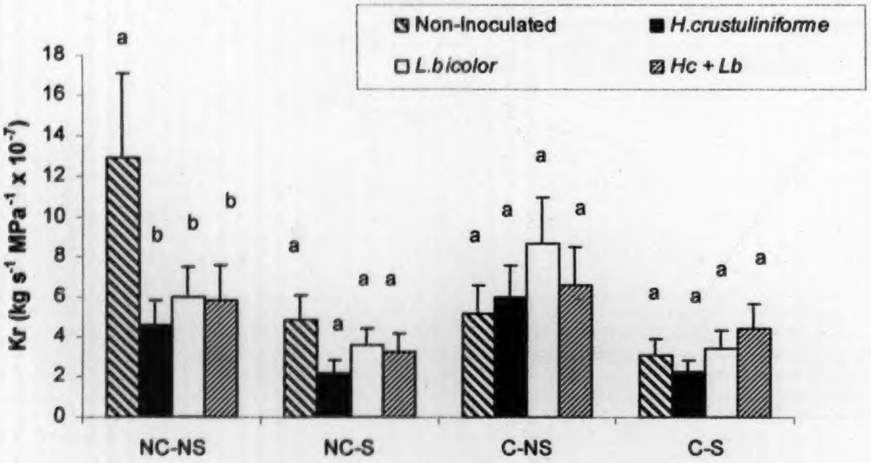

Figure 4.4. Root hydraulic conductance (K_r). The seedlings were either non-inoculated (N-I) or inoculated with *Hebeloma crustuliniforme* (Hc), *Laccaria bicolor* (Lb) or a mixture of *Hebeloma crustuliniforme* and *Laccaria bicolor* (Hc+Lb). Each group was subjected to soil compaction (C) or no compaction (NC) treatments and 0 mM NaCl (NS) or 60 mM NaCl (S). Means (n = 7) ± SE are shown.. Letters above the bars indicate significant differences ($\alpha = 0.05$) within each Compaction*NaCl treatment group (compaction-salt treatment combination within each inoculum treatment).

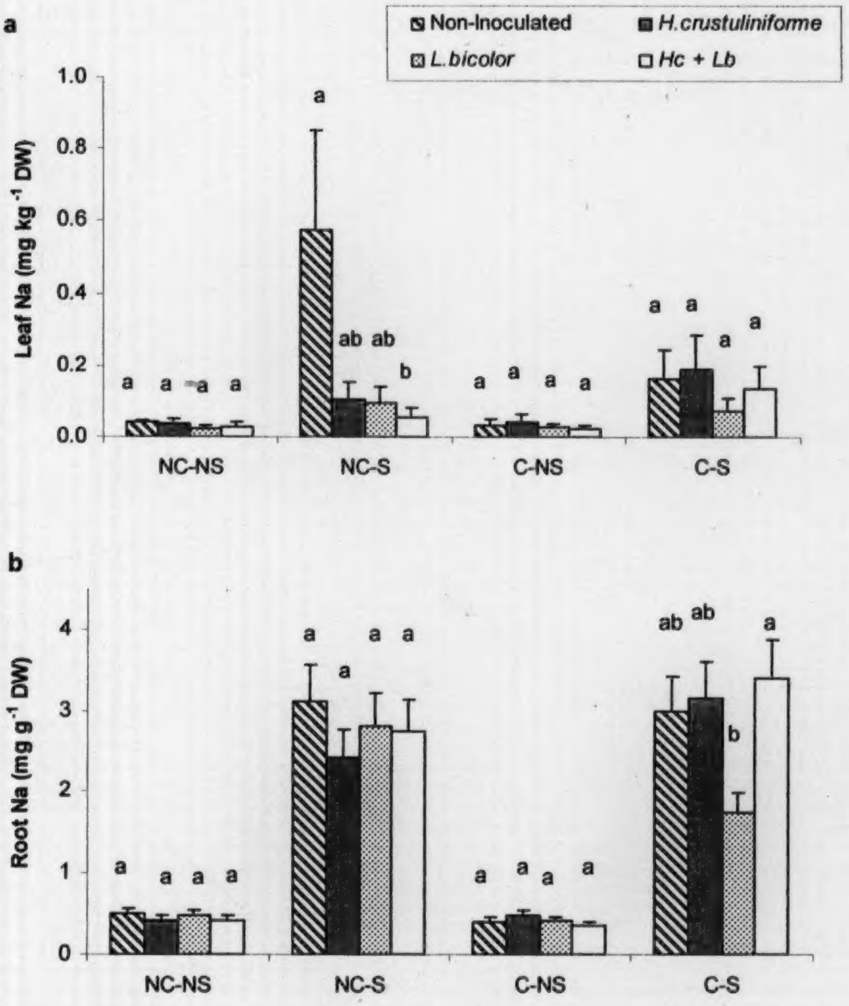

Figure 4.5. Leaf (a) and root (b) Na concentration in American elm seedlings. The seedlings were either non-inoculated (N-I) or inoculated with *Hebeloma crustuliniforme* (Hc), *Laccaria bicolor* (Lb) or a mixture of *Hebeloma crustuliniforme* and *Laccaria bicolor* (Hc+Lb). Each group was subjected to soil compaction (C) or no compaction (NC) treatments and 0 mM NaCl (NS) or 60 mM NaCl (S). Means (n = 7) ± SE are shown. Letters above the bars indicate significant differences ($\alpha = 0.05$) within each Compaction*NaCl treatment group (compaction-salt treatment combination within each inoculum treatment).

CHAPTER V: Effects of pH on NaCl resistance of ectomycorrhizal American elm (*Ulmus americana*) seedlings

5.1 Introduction

Soil pH is affected by the chemical composition of parent materials and has a major impact on nutrient availability and plant growth and distribution (Rengel 2002). Soil pH can change over time with progressing decomposition of organic matter and extrusion of organic acids and H^+ from the roots (Jahn and Palmgren 2002). However, human activities can result in rapid disturbances of soil pH. In urban areas, the problem is aggravated by importing soil with different pH and chemical characteristics and by the presence of soil pollutants which can alter plant responses to soil pH. In many study sites in Edmonton, AB, Canada, I commonly measured soil pH as high as 9.0 (Calvo Polanco and Zwiazek, unpublished data). In addition to high pH and soil compaction, high salt concentration is among the main soil factors affecting plant growth and survival in urban areas where salt is used as a de-icing agent.

The optimum pH for plant growth is species-dependent and varies from 3.5 to 8.5 (Larcher 2003). Nutrient uptake is often reduced at high pH. However, plants may modify the pH of their rhizospheres and increase alkalinity tolerance by extruding H^+ through the plasma membrane H^+-ATPase (Jahn and Palmgren 2002). Various environmental factors including wounding (Noubhani et al. 1996), salt (Kerkeb et al. 2001, Yang et al. 2007) and cold stress (Chelysheva et al. 1999) can alter the H^+-ATPase activity in plants and affect plant pH tolerance and salt resistance. Plant salt stress resistance involves metabolically-dependent membrane transport which controls Na cell concentrations and it is directly affected by the H^+-ATPase and Na^+/H^+ antiporter (Duby and Boutry 2008). Several studies have demonstrated an increase in H^+-ATPase activity of different herbaceous (Kerkeb et al. 2001, Sibole et al. 2005) and woody plants (Yang et al. 2007) associated with exposure to NaCl.

Soil pH tolerance of plants can be altered by the presence of ectomycorrhizal (ECM) associations (Siemens 2008). ECM fungi differ in their pH tolerance, over a wide range of pH (Kernaghan et al. 2002). In association with plants, *Hebeloma crustuliniforme* (Bull.) Quel. can tolerate alkaline sites (Hung and Trappe 1983) and *Laccaria bicolor* (R. Maire) Orton usually prefers acidic soils (McAfee and Fortin 1986). *Populus tremuloides* Michx. seedlings mycorrhizal with *H. crustuliniforme* showed increased alkalinity tolerance (Siemens 2008). Both *H. crustuliniforme* (Muhsin and Zwiazek 2002a, Bois et al. 2006, Nguyen et al. 2006) and *L. bicolor* (Bois et al. 2006, Nguyen et al. 2006) have also been shown to increase salt tolerance in plants by reducing Na uptake by plants. However, the exact mechanism of this reduction is unclear.

In the present study, I investigated the effects of pH treatments on NaCl tolerance in American elm (*Ulmus americana* L.) seedlings that had been inoculated with *H. crustuliniforme* and *L. bicolor* and compared them with non-inoculated control plants. *H. crustuliniforme* and *L. bicolor* are common ECM fungi that usually appear in the early stages of fungal succession in young forest stands and may represent pioneer mycorrhizal species (Smith and Read 1997). In my earlier study (Chapter IV in this thesis), I showed that these two ECM species can be beneficial to American elm seedlings growing in compacted salt-affected soils such as those commonly found in urban areas. I hypothesized that American elm seedlings inoculated with ECM fungi would increase their tolerance of pH extremes and improve their salt stress resistance.

5.2 Materials and methods

5.2.1 Plant material and growth conditions

American elm (*Ulmus americana* L.) seeds were collected at the University of Alberta campus, Edmonton, AB, Canada. The seeds were surface-sterilized with 3% H_2O_2 for 30s and germinated in Jiffy-7 pellets (Jiffy Products Ltd, Shippegan, NB Canada). The containers with germinating seedlings were placed in a growth room under the following growth conditions: 20/16°C day/night temperature, 16-h photoperiod, 65±10% relative humidity and 350 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at the seedling level. Four weeks after germination, the seedlings were transferred to 450 cm^3 pots (Kord Products Inc. Brampton, ON, Canada) and filled with a sterilized mixture of peat moss and sand (3:1 v/v). The soil mixture was sterilized by autoclaving twice for 20 min at 121°C with a 24-h interval. Commercial fertilizer (20:20:20 N:P:K) was added to seedlings at 1g L^{-1} every two weeks and the soil was flushed weekly with de-ionized water to prevent ion accumulation.

5.2.2 Fungal inoculation

Laccaria bicolor (UAMH 8232*)*, *Hebeloma crustiliniforme* (UAMH 5247) were obtained from the University of Alberta Microfungus Collection and Herbarium.

Fungal cultures were prepared in a modified Melin-Norkans liquid medium (Mason 1980) for four weeks as previously described (Calvo Polanco et al. 2008). When one-month old and four-months old, the seedlings were inoculated with pure liquid cultures containing *L. bicolor*, *H. crustuliniforme* or a 1:1 (by volume) mixture of *L. bicolor* and H. *crustuliniforme*. A non-inoculated group of seedlings to which water was added served as a control. For the first inoculation, fungal cultures were diluted with deionized water (1:20, by volume) and then the Jiffy pellets containing the seedlings were soaked in 1 L of the diluted cultures for 24 h. For the second inoculation, two 5-cm-deep holes were made in the soil on both sides of each seedling and 10 ml of inoculum (prepared as above except that diluted only 1:1 (by volume) with water) was applied to the each hole. Roots were checked for fungal colonization at the end of NaCl and pH treatments. The ends of six lateral roots were removed from each of the 6 seedlings per treatment combination and preserved in ethanol-glycerol-water solution (4:3:3, by volume). Distal, 1-2-cm long, root segments were cleared with 10% KOH, stained with 5% black ink-vinegar solution (Vierheilig et al. 1998) and examined under a light microscope to determine the percentage of root segments colonized by mycorrhizal fungi (Brundrett et al. 1996).

5.2.3 NaCl and pH treatments

One month after the second inoculation, inoculated and non-inoculated groups, each containing 60 seedlings, was divided into three groups of 20 seedlings, and subjected to different pH treatments (pH 3, 6 and 9). Each pH group was further divided into two groups of 10 seedlings to receive either 0 mM (NaCl control) or 60 mM NaCl treatment. Solutions for pH treatments were prepared as previously described (Kamaluddin and Zwiazek 2004) with equimolar concentrations of H_2SO_4, NaOH, or Na_2SO_4 dissolved in distilled water. The low, pH 3, medium, pH 6 and high, pH 9, treatments were achieved by adding 24.5 mg H_2SO_4 L^{-1}, 35.5 mg Na_2SO_4 L^{-1} and 20.0 mg NaOH L^{-1} to distilled water. The treatments were applied for 4 weeks by immersing the pots in the solutions every three days for 24 h .The seedlings were fertilized by adding 1 g L^{-1} 20:20:20 (N:P:K) commercial fertilizer to treatment solutions every two weeks. The soil was flushed once a week to prevent ion accumulation.

5.2.4 Transpiration rates and leaf chlorophyll concentrations

After four weeks of treatments, transpiration rates (E) were measured in the uppermost fully developed leaves of eight seedlings per treatment combination (n = 8) starting at four hours following the onset of the photoperiod. A LI-1600 steady porometer (LI-COR Biosciences, Linconln, NE, USA) was used for the measurements. Leave areas were calculated with a LI-3000 leaf area meter (LI-COR Biosciences, Lincoln, NE, USA).

Leaf chlorophyll a and b concentrations were determined in 6 seedlings from each treatment combination (n=6). The leaves from each seedling were bulked and freeze-dried. Chlorophyll was extracted from 20 mg of the freeze-dried leaf samples with 8 ml dimethyl sulfoxide (DMSO). The samples were incubated for 26 h in an oven at 60°C and the centrifuged extracts were analyzed spectrophotometrically (Ultrospec III, Pharmacia LKB, Uppsala, Sweden). Total chlorophyll was calculated using the Arnon's equation (Šesták et al. 1971).

5.2.5 Dry weights and root hydraulic conductivity (L_p)

Shoot and root dry weights were determined for eight seedlings per treatment combination (n = 8). Roots and stems were dried in an oven at 65°C for 48 h. To determine shoot dry weights, leaves were freeze-dried for 48 h and their weights added to the weights of stems.

A high pressure flow meter (HPFM, Dynamax Inc., Houston, TX, USA) was used for the root hydraulic conductance (K_r) measurements (Muhsin and Zwiazek 2002b). The shoots of seven seedlings (n = 7) per treatment combination were excised 1.5 cm above the root collar and the roots attached via the cut stem to the HPFM. The measurements of K_r were carried out on roots pressurized up to 0.5MPa (Tyree et al. 1995). After the K_r measurements, the roots were thoroughly washed and their volumes measured using the volume displacement method (Voicu and Zwiazek 2004) for the determination of root hydraulic conductivity (L_p).

5.2.6 Tissue Na concentrations

Na tissue analyses were carried out in six seedlings treated with NaCl and pH solutions for four weeks. Roots and leaves were separated, dried, and ground in a mortar with liquid nitrogen. Ground tissue samples (0.5-1 g) were extracted using the sulfuric acid - hydrogen peroxide wet digestion method (Richards 1993). The concentration of Na in the tissue was determined with a Model AA880 Atomic Absorption Spectrophotometer (Varian Inc., Mississauga, ON, Canada).

5.2.7 Experimental design and statistical analysis

The experiment was a 4x3x2 complete randomized factorial design with three fungal treatments plus a control non-inoculated treatment, three pH level treatment (3, 6 and 9) and two salt level treatment (60mM NaCl and 0mM NaCl). The analysis of variance was performed using the Mixed Procedure of SAS (Version 9.1, SAS Institute Inc.; Cary, NC, USA).). The data for chlorophyll, root hydraulic conductance (L_p) and Na leaf and root tissue concentrations were transformed with a $\log_{10}$ function to meet the ANOVA assumptions of normality of distribution and homogeneity of variance. The transformed means and their standard errors were back-transformed for their representation in figures. Comparisons at α=0.05 were done based on the results from the pdiff option in Proc Mixed of SAS (Littell et al. 2006) to examine the effects of pH*NaCl interaction within each inoculation treatment (Non-Inocualted, *H. crustuliniforme*, *L. bicolor* and *H. crustuliniforme* + *L. bicolor*) and the effects of the pH*NaCl interaction at the different inoculation treatmens. ANOVA p-values for the main factors and their interactions are shown in Table 5.1.

The statistical model for the experiment was as follows:

$$Y_{ijkl} = \mu + M_i + N_j + P_k + M_iN_j + M_iP_k + N_jP_k + M_iN_jP_k + e_{ijkl}, \text{ where}$$

Y_{ijkl} = value of individual observation (*i* = mycorrhiza, *j* = NaCl, *k* = pH *l*=observation)

μ = overall mean of observations

M_i = effect of i^{th} treatment (i = mycorrhizal treatment)

N_j = effect of j^{th} treatment (j = NaCl treatment)

P_k = effect of k^{th} treatment (k= pH treatment)

M_iN_j = interaction effect of i^{th} and j^{th} treatments

M_iP_k = interaction effect of i^{th} and k^{th} treatments

N_jP_k= interaction effect of j^{th} and k^{th} treatments

$M_iN_jP_k$= interaction effect of i^{th}, j^{th} and k^{th} treatments

e_{ijkl} = residual error

5.3 Results

5.3.1 Root colonization, plant dry weights and leaf chlorophyll concentrations

Although roots of inoculated plants were largely covered with a mass of loosely-organized hyphae growing in the surface of the roots, they did not develop a well defined mantle or Hartig net. The leaves of inoculated seedlings became chlorotic during the first month following the first inoculation, but recovered before pH and NaCl treatments were applied.

Fungal inoculation and pH treatment had no significant overall effects on the total dry weights of plants and there was no interaction between inoculation, salt and pH treatment (Figure 5.1a, Table 5.1). However, in the pH 6 treatment group the dry weights of plants inoculated with *H. crustuliniforme* and not treated with NaCl were higher compared with seedlings inoculated with both *H. crustuliniforme* and *L. bicolor*. The small reductions in dry weights that were observed as a result of 60 mM NaCl treatments in inoculated and non-inoculated plants were not significant at $\alpha = 0.05$ (Figure 5.1a).

In the absence of NaCl treatment, fungal inoculation had a significant on non-treated plants at pH 6 on shoot to root dry weight ratios (Figure 5.1b). In plants treated with 60 mM NaCl, shoot to root ratios were significantly higher in inoculated plants compared with non-inoculated seedlings at low ($p = 0.036$) but not high ($p = 0.754$) pH treatment (Figure 5.1b). At medium pH, the overall effect of inoculation on shoot to root ratios was not significant ($p = 0.156$), but there was a significant increase in shoot to root ratios for plants inoculated with *L. bicolor* compared with the non-inoculated plants (Figure 5.1b).

Treatment with 60 mM NaCl reduced leaf chlorophyll concentrations in non-inoculated seedlings compared with inoculated plants and the greatest, two-fold, decrease ($p < 0.001$) occurred at pH 6 (Figure 5.1c). In the absence of NaCl treatment, leaf chlorophyll concentration at pH 6 and 9 in seedlings inoculated with *H. crustuliniforme* + *L. bicolor* were lower compared with those in other inoculation treatments (Figure 5.1c).

5.3.2 Gas exchange (E) and root hydraulic conductivity (L_p)

For all inoculation groups considered together, pH did not significantly affect transpiration rates (E) (Figure 5.2a, Table 5.1). In non-inoculated seedlings, NaCl treatment reduced E at pH 6 and 9, but not at pH 3 (Figure 5.2a). At pH 6, E was about two-fold lower in NaCl-treated non-inoculated plants compared with non-treated controls ($p = 0.001$) (Figure 5.2a). For *H. crustuliniforme*- and *L. bicolor*-inoculated plants, the greatest reduction of NaCl was at pH 9 (Figure 5.2a).

Similarly to E, pH had little effect on root hydraulic conductivity (L_p) except in the presence of NaCl (Figure 5.2b). The effects of inoculation on L_p varied at different pH levels and for different inoculation treatments. In the absence of NaCl, L_p was higher in inoculated plants compared with non-inoculated seedlings at pH 6 (Figure 5.2b). At pH 9 NaCl treatment decreased L_p by up to three-fold in non-inoculated ($p = 0.043$) and *H. crustuliniforme* ($p = 0.01$) seedlings (Figure 5.2b).

5.3.3 Na tissue concentrations

For all inoculation groups and pH treatments, roots treated with NaCl accumulated less Na compared with leaves (Figure 5.3a,b). At pH 3, roots of inoculated seedlings treated with 60 mM NaCl had more than 50% higher Na concentrations compared with the roots of non-inoculated plants ($p = 0.015$) (Figure 5.3a). However, this difference was not present at pH 6 and 9 (Figure 5.3a).

There was no inoculation effect on Na leaf concentration in plants treated with NaCl at pH 4 (Figure 5.3b). At pH 6 and 9, Na leaf concentrations were significantly reduced in plants inoculated with *H. crustuliniforme* + *L. bicolor* ($p = 0.047$) and *H. crustuliniforme* ($p = 0.023$) respectively (Figure 5.3b).

5.4 Discussion

Inoculation of American elm seedlings with ECM fungi did not have a large effect on seedling dry weights for any of the studied pH and NaCl treatments (Figure 5.1a). Therefore, it appears that, unlike in an earlier study (Calvo Polanco et al. 2008), American elm seedlings were able to supply *H. crustuliniforme* and *L. bicolor* with the required resources without affecting their own growth rates. Although the seedlings initially responded to fungal inoculation with leaf chlorosis, they recovered before the commencement of pH and NaCl treatments.

Leaf chlorophyll concentration was differently affected by pH in different inoculation groups. When treated with 60 mM NaCl, leaves of inoculated plants had higher chlorophyll concentrations compared with non-inoculated seedlings, but only at pH 6. In the absence of salt, chlorophyll concentration in the leaves of plants inoculated with *H. crustuliniforme* + *L. bicolor* was higher at pH 3 compared with pH 6 and 9. Leaf chlorosis can be often caused by Fe deficiency at high pH (Mengel 1994). However, high pH did not have an effect on chlorophyll concentrations in non-inoculated plants (Figure 5.1c). It appears that the factors which are responsible for the differences in chlorophyll concentrations between various treatments may be related to complex nutritional and metabolic factors.

Shoot to root ratios were higher in the inoculated plants compared with non-inoculated seedlings when the plants were treated with NaCl at pH 3 and 6 (Figure 5.1b). These results suggest that NaCl affected root dry weights more than shoot dry weights in inoculated plants and that this response was pH-dependent. Similarly, the differences in E and L_p between NaCl-treated inoculated and non-inoculated plants appeared to be greater at pH 3 and 6, but not at pH 9 (Figure 5.2 a,b). Therefore, both ECM associations had a greater ability to cope with the physiological effects of NaCl at the lower soil pH which cannot be explained by the differences in Na leaf concentrations (Figure 5.3b).

In *Betula papyrifera* Marsh. stomatal conductance and root hydraulic conductivity were reduced by both high (9) and low (3) pH compared with pH 6 and these effects were thought to be mediated by the effect of pH on the aquaporin-mediated transport (Kamaluddin and Zwiazek 2004). It is interesting that in the present study, the only significant increases in L_p by ECM in the absence of NaCl was also at pH 6 suggesting a possible role of the aquaporin-mediated cell-to-cell transport (Figure 5.2b). In the presence of NaCl, ECM plants had higher Lp compared with non-inooculated seedlings at pH 3 and 6. In *Populus tremuloides* seedlings, inoculation with *H. crustuliniforme* resulted in an increase of L_p at pH 7 and this increase did not appear to be due to the aquaporin-mediated water transport (Siemens 2008). The increase in L_p of ECM plants may involve both increased aquaporin expression (Marjanović et al. 2005) and enhanced apoplastic transport (Muhsin and Zwiazek 2002b). Therefore, the effects of pH on L_p responses in ECM plants may be complex and involve different water flow pathways.

Plant injury and physiological responses are usually directly proportional to Na tissue concentrations (Franklin and Zwiazek 2004). It appears that an overall ability of roots in American elm seedlings to store Na is lower compared with other studied plants. As shown in this and in an earlier study (Calvo Polanco et al. 2008), elm roots accumulated less than 3 mg g^{-1} DW Na. This compares with 6-17 mg g^{-1} DW Na reported for *Picea glauca* (Muhsin and Zwiazek 2002a, Franklin and Zwiazek 2004), *Pinus banksiana* (Apostol et al. 2004), *Cornus stolonifera* (Renault et al. 2001), and *Populus tremuloides* (Yi et al. 2008). Consequently, most of the Na in this study was deposited in the leaves (Figure 5.3b). Prevention of Na transport into the shoots is among the most effective salt resistance mechanisms used by plants (Shannon et al. 1994, Renault et al. 2001) and thought to be affected by metabolic factors (Drew and Läuchli 1985, Redfield et al. 2003). However, it was also proposed that salt sequestration in roots may be more important for evergreen conifers compared with deciduous angiosperm trees since deciduous trees can use leaf shedding as an effective salt exclusion mechanism (Yi et al. 2008).

The seedlings inoculated with the ECM fungi had higher Na root concentrations than non-inoculated plants when treated with 60 mM NaCl at pH 3, but not at pH 6 and 9 (Figure 5.3a). An opposite trend was observed for the leaves where ECM associations reduced Na concentrations at pH 3 and 6, but not at 9 (Figure 5.3b). It is interesting that *H. crustuliniforme* was effective in reducing Na leaf concentration at pH 9, but not pH 3 and 6. Contrary to *L. bicolor*, which prefers acidic soils (McAfee and Fortin 1986), H. *crustuliniforme* has been previously found to have high alkalinity tolerance (Hung and Trappe 1983, Siemens 2008). It has been demonstrated that H^+-ATPase activity in plants is increased by NaCl (Kerkeb et al. 2001, Sibole et al. 2005, Yang et al. 2007). Sodium sequestration in plant tissues involves metabolically-dependent Na membrane transport which is driven by the H^+-ATPase-generated H^+ gradient across the membrane (Kerkeb et al. 2001). In roots, soil pH directly affects H^+ gradient and membrane transport properties and therefore, may alter Na transport across the plasma membrane. Also, H^+-ATPase activity has a pH optimum of approximately 6.5, which can be shifted upward by the lipid composition of the membrane (Palmgren 1988). Membrane structure and composition may be altered by external pH, therefore, H^+-ATPases with different pH optima may play a role in conferring acid or alkaline tolerance to organisms (Axelsen et al. 1999). Although ECM associations have not been studied with respect to their effects on H^+-ATPase activity, in plants forming vesicular-arbuscular mycorrhizas, plasma membrane ATPase is thought to be under strong mycorrhizal control, likely resulting from increasing the coupling efficiency of H^+ transport and ATP hydrolysis (Benabdellah et al. 1999, Bago et al. 1997).

Several studies showed reductions in Na (Mushin and Zwiazek 2002a, Nguyen et al. 2006, Langenfeld-Heyser et al. 2007, Calvo Polanco et al. 2008) and Cl (Nguyen et al. 2006) tissue concentrations of ECM associations. However, the exact mechanisms of these reductions are not known. It has been proposed that the fungal sheath of ectomycorrhizal roots may increase root hydrophobicity and reduce the uptake of cations by roots from the soil (Bücking et al. 2002). However, in the present and earlier (Calvo Polanco et al. 2008) studies, Na leaf concentrations in inoculated elm seedlings was reduced in the absence of a corresponding reduction in the roots and in the absence of a well defined ECM mantle.

In conclusion, inoculation of American elm seedlings with ECM fungi did not have a large effect on seedling dry weights for any of the studied pH and NaCl treatments. However, when the inoculated seedlings were treated with 60 mM NaCl at pH 3 and 6, shoot to root ratios and L_p were higher compared with non-inoculated plants, possibly reflecting changes in water flow properties. Root Na concentrations were higher in inoculated plants at pH 3 compared with non-inoculated seedlings. However, there was no effect of inoculation on root Na concentrations at pH 6 and 9. Fungal inoculation affected leaf Na concentrations differently at different pH levels. The results point to the importance of soil pH for salt resistance of mycorrhizal plants.

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Table 5.1. ANOVA p-values for the main factors and their interactions. The main factors are: inoculation (non-inoculated seedlings or inoculated with *H. crustuliniforme*, *L. bicolor* or *H. crustuliniforme* + *L. bicolor*), NaCl (0 and 60 mM NaCl treatments) and pH (3, 6 and 9). The measured parameters includes total dry weights (DW), shoot to root ratios (S:R ratio), leaf chlorophyll concentrations (Chl), transpiration rates (E), hydraulic conductivity (L_p) and root and leaf Na concentrations

	Total DW	S:R ratio	Chl	E	L_p	Root Na	Leaf Na
Inoculation	0.509	0.020	0.141	0.447	0.093	0.095	0.006
pH	0.799	0.143	0.309	0.503	0.878	0.206	0.360
Inocualtion*pH	0.487	0.766	0.525	0.572	0.192	0.107	0.839
NaCl	0.005	0.144	<0.001	<0.001	0.002	<0.001	<0.001
Inoculation*NaCl	0.727	0.281	0.460	0.697	0.094	0.025	0.297
pH*NaCl	0.384	0.212	0.618	0.458	0.069	0.025	0.013
Inoculation*pH*NaCl	0.995	0.547	0.203	0.430	0.889	0.010	0.402

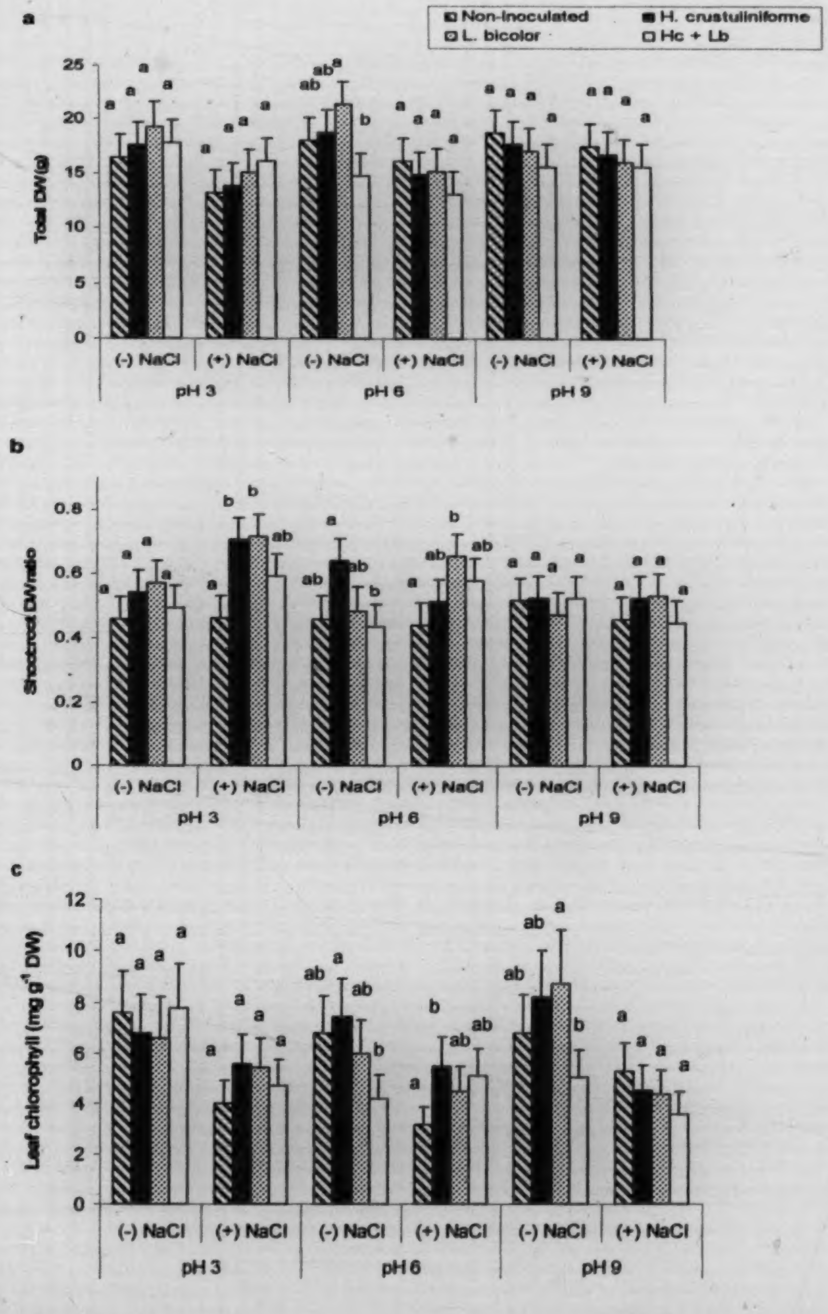

Figure 5.1. Total dry weights (a), shoot to root ratios (b) and leaf chlorophyll concentrations (c) in non-inoculated American elm seedlings and seedlings inoculated with *Hebeloma crustuliniforme*, *Laccaria bicolor* and a mixture of *H. crustuliniforme* + *L. bicolor*. The plants were treated with 0 [(-) NaCl] or 60 mM NaCl [(+) NaCl] and with pH 3, 6 and 9 solutions for four weeks. Means (n = 8) + SE are shown. Different letters above bars indicate significant differences within each treatment group at α = 0.05.

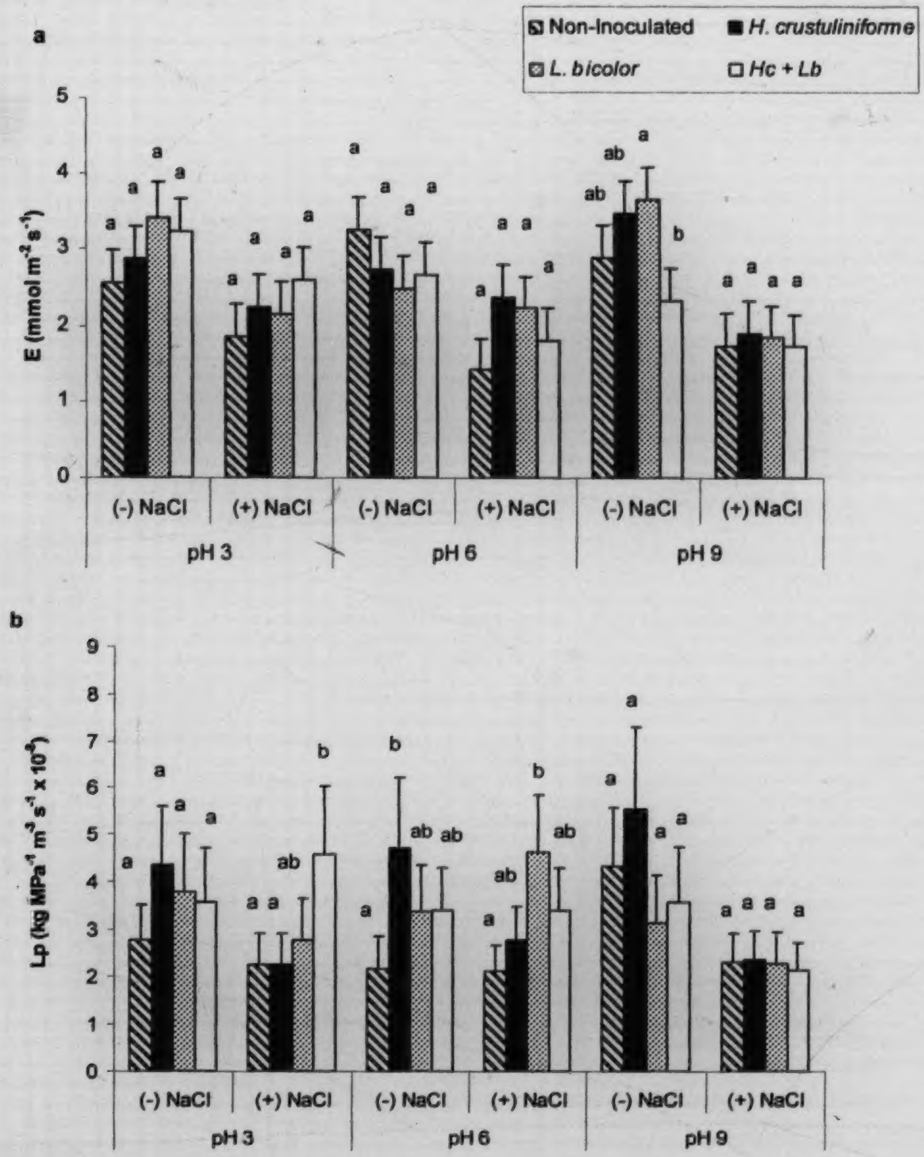

Figure 5.2. Transpiration rates (E) (a) and root hydraulic conductivity (L_p) (b) in non-inoculated American elm seedlings and seedlings inoculated with *Hebeloma crustuliniforme*, *Laccaria bicolor* and a mixture of *H. crustuliniforme* + *L. bicolor*. The plants were treated with 0 [(-) NaCl] or 60 mM NaCl [(+) NaCl] and with pH 3, 6 and 9 solutions for four weeks. Means (n = 8 for E and n = 7 for L_p) + SE are shown. Different letters above bars indicate significant differences within each treatment group at $\alpha = 0.05$.

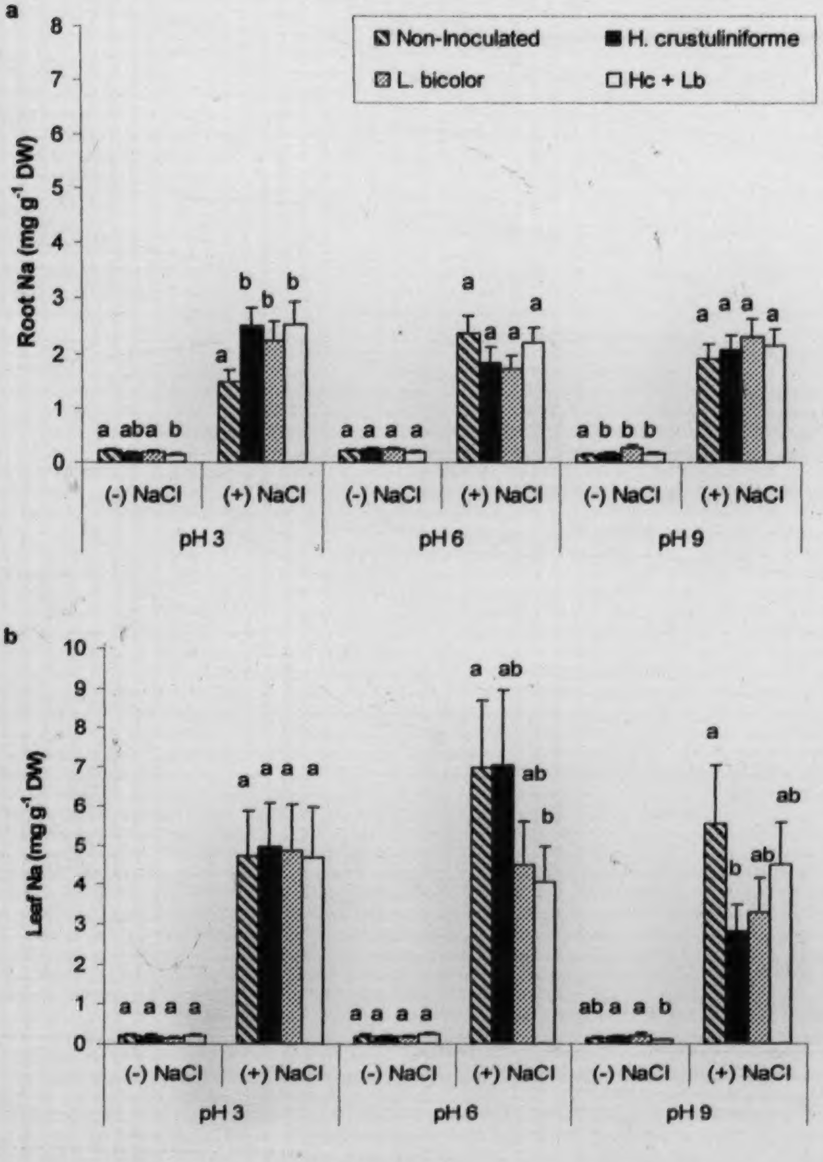

Figure 5.3. Root (a) and shoot (b) Na concentrations in non-inoculated American elm seedlings and seedlings inoculated with *Hebeloma crustuliniforme*, *Laccaria bicolor* and a mixture of *H. crustuliniforme* + *L. bicolor*. The plants were treated with 0 [(-) NaCl] or 60 mM NaCl [(+) NaCl] and with pH 3, 6 and 9 solutions for four weeks. Means (n = 6) +SE are shown. Different letters above bars indicate significant differences within each treatment group at $\alpha = 0.05$.

CHAPTER VI: General discussion and conclusions

6.1 General discussion and conclusions

Effects of salt on plants have been extensively studied. However, most of these studies have not considered other environmental factors that often accompany soil salinity and that may affect plant responses.I considered for this study the main environmental factors that could potentially have a major impact on plant salt responses in the areas affected by oil sands mining and urban activities. Mycorrhizal associations are among the most significant biotic factors that alter responses of plants to salt (Bois et al. 2006, Nguyen et al. 2006). Since most of tree species have mycorrhizal associations, their responses to salt and other environmental factors should be studied in the presence of these associations.

Results of this study are presented in four experimental chapters. Chapter II examined the effects of KF on NaCl resistance of jack pine (*Pinus banksiana*), black spruce (*Picea mariana*) and white spruce (*Picea glauca*) seedlings colonized by the mycorrhizal fungus *Suillus tomentosus*. In black spruce and white spruce, but not in jack pine, treated with 60 mM NaCl + 5 mM KF, needle necrosis and shoot F concentrations were reduced compared with the 5 mM KF treatment (Chapter II). Although this effect does not appear to be related to the reduction in transpiration rates by NaCl, it would be interesting to investigate whether it is caused by osmotic effect or ionic toxicity of NaCl. Also, another study should compare responses of mycorrhizal and non-mycorrhizal plants to fluoride and NaCl. The key missing knowledge required to interpret the mechanisms of salt stress protection by ectomycorrhizal fungi is the exact distribution of NaCl within the mycorrhizal association.

Interestingly, a similar reduction was not observed for Cl suggesting a possible competitive inhibition of F transport by Cl in both spruce species. Since it has been suggested that F is transported across cell membranes partly in an uncharged form of HF (Kronberger 1988), I speculated that, similarly to many other small uncharged molecules (Maurel 2007), the transmembrane transport of fluoride may also involve aquaporins. Since, the activity of aquaporins is strongly inhibited by NaCl (López-Berenguer et al. 2006, Martínez-Ballesta et al. 2006), this could help explain the reduction of F tissue concentrations in white spruce and black spruce seedlings treated with 5 mM KF + 60 mM.

The examined hypothesis for this study was that fluoride would alter NaCl resistance of ectomycorrhizal jack pine, black spruce and white spruce seedlings. However, the lack of effect of NaCl on F shoot concentrations in jack pine suggests that the transport mechanisms of F may vary between plant species and (or) that these properties were differently affected by *S. tomentosus* in jack pine compared with the studied spruce species.

Chapter III examined how ectomycorrhizal fungi (*Hebeloma* sp., *Suillus tomentosus*, and *Wilcoxina mikolae* var. *mikolae*) would affect the responses of jack pine seedlings to 2 mM H_3BO_3 in the presence and absence of 60 mM NaCl treatment. Elevated B levels are often present in saline soils, particularly in areas with poor drainage or with shallow water tables that limit opportunities for leaching (Gupta et al. 1985, Renault et al. 1998). Boron may aggravate salt injury in plants and enhance Na concentration in the shoots (Apostol et al. 2002). Similarly to NaCl, the ability of plants to tolerate elevated soil B levels largely depends on the restriction of uptake and sequestration of B in less sensitive parts of the plant (Nable et al. 1997). In *Pinus banksiana* (Apostol et al. 2002), *Eucalypus sp.* (Marcar et al. 1999) and *Zea mays* (Bastías et al. 2004) B uptake was reduced by NaCl treatment. In the present study, mycorrhizal plants had lower shoot Na concentrations compared with non-inoculated jack pine seedlings when treated with NaCl. Shoot Cl concentrations were reduced in mycorrhizal plants treated with NaCl, but higher in inoculated seedlings compared with the non-inoculated plants treated with NaCl +B. However, the differences in B shoot concentration between the 2 mM B and 2 mM B + 60 mM NaCl treatments were relatively small. Similarly, there was little effect of mycorrhizal fungi on B root and shoot concentrations suggesting that the applied B treatment was not a major factor in this experiment affecting the responses of plants to NaCl. This is a puzzling result that is in contrast to earlier studies that showed a reduction in shoot B concentrations in the presence of NaCl (Marcar et al. 1999, Bastías et al. 2004), including a study with jack pine which used the same boron treatment concentration (Apostol et al. 2002). It was also interesting that in the present study, there was no effect of mycorrhizal inoculation on boron tissue concentrations. Future studies should include treatments with different boron concentrations because boron toxicity likely varies between plants of the same species depending on factors such as age of plants and experimental conditions. Similarly to other laboratory experiments, it would be important to validate the results in field studies.

In Chapter IV, the effects of soil compaction were studied in mycorrhizal and non-mycorrhizal American elm seedlings exposed to NaCl stress. Soil compaction decreases pore size and reduces soil oxygen content, effectively creating hypoxic conditions for roots (Mariani et al. 2006; Haeussler and Kabzems 2005). Therefore, soil compaction may potentially affect salt resistance of plants. Hypoxia may increase the entry of Na into roots and allow more NaCl to move into the xylem apoplastically (Barrett-Lennard et al. 2003). Root hypoxia also inhibits the function of root aquaporins and reduces root water uptake (Zhang and Tyerman 1991). Since the diameter of fungal hyphae is smaller than the diameter of the finest roots, I speculated that hyphae would more easily penetrate small pores in compacted soils and access the water and nutrient resources (Schack-Kirchner et al. 2000, Allen 2007). The reduction in Na uptake by ectomycorrhizal associations has been reported in other studies (Bois et al 2006, Nguyen et al. 2006, Chapter III and V). In this study, ECM fungi reduced leaf Na uptake by elm seedlings when treated with NaCl in non-compacted soil. However, contrary to my original hypothesis, soil compaction reduced leaf Na concentrations in both NaCl-treated non-mycorrhizal and mycorrhizal plants. The results suggest that soil compaction affected plants differently from root hypoxia that was created by reduced oxygen levels in solution culture experiments where the ability of plants to sequester Na was reduced (Apostol and Zwiazek 2003; Redfield et al. 2003). My interpretation of the results included a possibility that the reduction of plant water uptake (observed reductions in transpiration rates and root hydraulic conductance) together with lower growth rates could have contributed to the reduced Na transport to the leaves.

In Chapter V, I studied the effects of pH on the responses of mycorrhizal (inoculated with *Hebeloma crustuliniforme* and *Laccaria bicolor*) and non-mycorrhizal American elm seedlings to NaCl. Soil pH is among the most important factors affecting root ion uptake. The optimum pH for plant growth is species-dependent and varies from 3.5 to 8.5 (Larcher 2003). The tolerance of soil pH can be increased by the presence of mycorrhizal associations (Slankis 1974, Medeiros et al. 1994). Mycorrhizas have been demonstrated to increase growth (Aggangan et al. 1996) and root hydraulic conductivity under pH extremes (Siemens 2008). Therefore, it is conceivable that the presence of ectomycorrhizal associations may alter the effects of soil pH on salt responses in plants. In my study, fungal inoculation affected root and leaf Na concentrations differently at different pH levels. Higher Na root concentrations were found in inoculated compared with non-inoculated plants when treated with 60 mM NaCl at pH 3, but not at pH 6 and 9. An opposite trend was observed for the leaves where ectomycorrhizal associations reduced Na concentrations at pH 3 and 6, but not at 9. There were also differences in plant responses to NaCl depending on the applied inoculum. *Hebeloma crustuliniforme* was effective in reducing Na leaf concentration at pH 9, but not pH 3 and 6. *H. crustuliniforme* has been previously found to have high alkalinity tolerance (Hung and Trappe 1983, Siemens 2008), in contrast to *L. bicolor*, which prefers acidic soils (McAfee and Fortin 1986). Sodium sequestration in plant tissues involves metabolically-dependent Na membrane transport which is driven by the by the H^+-ATPase-generated H^+ gradient across the membrane (Kerkeb et al. 2001). Although ectomycorrhizal associations have not been studied with respect to their effects on H^+-ATPase activity, in plants forming vesicular-arbuscular mycorrhizas, plasma membrane ATPase is thought to be under strong mycorrhizal control, likely resulting from increasing the coupling efficiency of H^+ transport and ATP hydrolysis

(Benabdellah et al. 1999, Bago et al. 1997). Therefore, it is possible that H^+-ATPase may be involved in the responses of ectomycorrhizal plants to pH.

The results of the soil compaction and pH studies only partly supported the studied hypothesis. In the future work, other plant species should be considered. Although American elm can be ectomycorrhizal, it mostly forms VAM associations (Brundett et al. 1990) and perhaps did not respond in the same manner to the applied treatments as would typically ectomycorrhizal plant species. Elm trees are well adapted to various soil conditions including poor drainage and wide range of soil pH (Bey 1990). It is difficult to control soil pH and maintain constant soil properties in the controlled-environment studies. This research should be extended in the future to include field studies where these conditions are already present.

The concept of increasing stress resistance of plants with mycorrhizas has attracted a lot of attention. Since the pioneering studies of Harley, McCready and co-workers in the 1950s and 1960s which demonstrated that ectomycorrhizal associations help with the uptake of nutrients from the soil (Harley and Smith 1983), evidence has accumulated pointing to possible roles of ectomycorrhizas in protecting plants from metal toxicity (Jones and Hutchinson 1988, Jentschke and Godbold 2000), salt (Bois et al. 2006, Nguyen et al. 2006, Yi et al. 2008), drought (Morte et al. 2001, di Pietro et al. 2007), pH extremes (Kernaghan et al. 2002, Siemens 2008), and pathogens (Morin et al. 1999, Robin 2001). However, the benefits of ectomycorrhizal associations also come at a cost to the plant. In Chapter IV, American elm seedlings responded to fungal inoculation with growth and leaf chlorophyll content reductions. Growth reductions have been frequently reported for ectomycorrhizal associations (Corrêa et al. 2006, Karst et al. 2008) but they may only be temporary. The effects of ectomycorrhizal associations on plant physiological responses have been studied with relatively few species of fungi. The present studies demonstrate that plants inoculated with different species of mycorrhizal fungi respond differently to the various soil factors. In terms of NaCl resistance, all plant species that were used in the present study benefited from the different mycorrhizal fungi that they were inoculated with, even in the absence of clearly visible ectomycorrhizal structures. However, there are many stress factors that interact in the field with each other and with salt. The present project was an initial attempt to take some of these factors into account, separately and under controlled conditions to understand these possible interactions. More work will be required to extend these findings to the field conditions. Although this research is not part of the present PhD project, field studies in the City of Edmonton and oil sands reclamation sites have already commenced.

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